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THE STABILITY OF BACTERIAL SUSPENSIONS.

IV. The Combination of Antigen and Antibody at Different Hydrogen Ion Concentrations.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, September 21, 1922.)

INTRODUCTION.

In a preceding paper (1) it was observed that the agglutinating power of normal and immune serum increased as the pH approached the acid agglutination optimum of the bacterium under test. Several hypothetical explanations of this phenomenon suggest themselves: (*a*) The agglutinating power of antibody might be enhanced by incubation at high C_H (4.0 to 5.0); (*b*) the maximal combination of antigen and antibody might take place at this zone; (*c*) the same amount of antibody in combination might have a greater effect in this range; or (*d*) the other constituents of the serum may have an agglutinating influence in this range. The following experiments show that the first two suggestions are untenable, and that the third hypothesis accounts for the observations.

Method.

The organism used in these experiments was *Bacillus typhosus*, Pfeiffer strain, obtained through the kindness of Dr. Charles Krumwiede of the Department of Health of the City of New York, to whom we are also indebted for the high titer antityphoid agglutinating serum. The serum dilutions were carried out in glycocoll-Na acetate- Na_2HPO_4 buffer mixtures of definite pH, described in the preceding paper (1). The "standard" bacterial suspensions were prepared as described in the preceding paper (1). Suspensions of higher concentration were used for absorption and other tests, and are always referred to the standard suspensions, as follows: A 10 $\times$ *T*

suspension would be one in which the bacteria from 150 cc. of broth were suspended in *15 cc.* of distilled water. A $20 \times T$ suspension would be made by suspending the bacteria from 150 cc. of media in *8 cc.* of distilled water, and so on.

Experiment 1. Agglutinating Power of Antityphoid Serum Kept at Various C_H *for 2 Hours at 43°C.*—0.1 cc. quantities of serum > typhoid were diluted with 2.9 cc. of glycocoll-acetate-phosphate buffer mixtures (M/12) of various pH from 9.9 to 2.0. Dilution = 1:30. The tubes containing these dilutions were placed in the water bath at 43°C. for 2 hours. 0.1 cc. of each sample was then diluted with 3.35 cc. of buffer mixture pH 6.0. Colorimetric test showed all of the mixtures to be at or very near to pH 6.0. Dilution now approximately 1:1,000. Each cc. contained 0.001 of immune serum. Each sample was now diluted by 2's with buffer solution pH 6.0 to a point where 3.7×10^{-6} cc. of the original immune serum was present in each tube. 1 cc. of standard 4 times washed typhoid suspension was now added to each series of dilutions and incubated at 43°C. for 14 hours. The results are summarized in Table I.

It will be observed from Table I that over a range from pH 9.9 to 3.9, 43°C. for 2 hours has very little effect on the subsequent agglutinating power of antityphoid serum. It is true that a slight decrease in activity manifests itself in Tubes 4 and 5 (pH 6.0 and 5.0). This difference is probably not adventitious, since subsequent tests confirmed the observation. It is probably due to precipitation of the globulin. A really perceptible deleterious effect is observed, however, in Tubes 9 and 10 (pH 2.7 and 2.2). Here a decrease of eightfold loss of agglutinative power is observed in Tube 9, and sixteenfold in Tube 10. The experiment demonstrates that hydrogen ion concentrations of pH 5.0 and 4.6 do not have an enhancing effect on the agglutinating power of the serum, and the first hypothesis has, therefore, to be rejected.

A second explanation of the increased agglutinating activity of serum at pH 4.5 to 5.0 lies in the idea that the combination between antigen and antibody might be more complete in this zone of hydrogen ion concentration. This would be the assumption, *a priori*, of those who believe that the combination of antigen and antibody is influenced by electrical forces (2). Assuming that antibody behaves in a manner similar to serum protein, the former should carry a slight *positive* charge at pH 4.6. The bacterium, on the other hand, is

negatively charged at this point (1). Therefore, if union between bacterium and antibody is a matter of neutralization of opposite charge, it should be more complete at pH 3.5 to 4.5, where the organism and antibody are oppositely charged, than at pH 6.0 or 7.0, where both bacterium and antibody would be negatively charged.

TABLE I.

Agglutinating Power of Antityphoid Serum Kept at Various C_H for 2 Hours at 43°C.

Tube.	pH at which serum was kept for 2 hrs. at 43°C.	Serum diluted from various pH to pH 6.0; 43°C., 14 hrs., + typhoid suspension. Cc. of serum per cc. of solution.*								
		1×10^{-1}*	5×10^{-1}	2.5×10^{-1}	1.2×10^{-1}	6×10^{-2}	3×10^{-2}	1.5×10^{-2}	7×10^{-2}	3.5×10^{-2}
1	9.9	C.	C.	C.	C.	C.	++	+	Tr.	Tr.
2	9.0	C.	C.	C.	C.	C.	++	+	−	−
3	7.5	C.	C.	C.	C.	C.	++	+	Tr.	−
4	6.0	C.	C.	C.	C.	++	+	Tr.	−	−
5	5.0	C.	C.	C.	C.	++	++	+	Tr.	−
6	4.6	C.	C.	C.	C.	C.	++	+	Tr.	−
7	3.9	C.	C.	C.	C.	C.	++	+	+	Tr.
8	3.3	C.	C.	C.	C.	++	++	+	+	Tr.
9	2.7	C.	C.	++	+	−	−	−	−	−
10	2.2	C.	++	+	−	−	−	−	−	−
11	Control serum undiluted; 43°C.; 2 hrs.	C.	C.	C.	C.	C.	++	+	−	−

*Represents concentration of serum $\left(i.e. \frac{1}{\text{dilution}} = \text{cubic centimeter of serum per cubic centimeter of solution}\right)$. Subsequent addition of equal volume of suspension divides this concentration by 2.

The degree of combination is best studied by absorption experiments. Since the absorption of antibody is carried out with concentrated suspensions, it was considered necessary to determine the relationship existing between concentration of the microbic suspension and the amount of serum necessary to cause complete agglutination.

Experiment 2. Relation between Concentration of Bacterial Suspension and Complete Agglutinating Dose of Typhoid Immune Serum.—As described above, the standard suspension used in these typhoid agglutination experiments is pre-

pared by suspending the 4 times washed organisms from 150 cc. of a 16 hour broth culture in 160 cc. of distilled water. Five suspensions were now prepared. Washed organisms from 150 cc. of broth were suspended in 8, 16, 32, 64, and 160 cc. of distilled water, thus securing suspensions of 20, 10, 5, and 2.5 times the concentration of the original. 1 cc. of each of these suspensions was now tested against a series of dilutions of antityphoid serum. Glycocoll-acetate- phosphate buffer mixture, pH 6.3, was used as diluent. Incubation at 43°C. for 14 hours. "Complete agglutination" was considered to take place when the supernatant fluid was water-clear, no whorl of suspended bacteria being visible when the tube was agitated before a strong beam of light. "++" was read when a heavy sediment was present, with a still cloudy supernatant fluid showing a distinct whorl of unagglutinated organisms. The results are summarized in Table II.

TABLE II.

Relation between Concentration of Suspensions and Complete Agglutinating Dose of Antityphoid Serum.

Concentration of *T* suspension.	Concentration of antityphoid serum $\times 10^{-3}$=					
	150	75	37.5	18	9	4.5
20 times.	C.	++	+	+	Tr.	Tr.
10 "	C.	C.	++	++	++	+
5 "	C.	C.	C.	++	++	+
2.5 "	C.	C.	C.	C.	++	++
1	C.	C.	C.	C.	C.	++

Table II demonstrates that within the limits of suspension concentration studied in this experiment, the amount of serum necessary to agglutinate varies directly with the concentration of the suspension. The results show that at least 16 times less serum is required to agglutinate completely the standard suspension than to cause the same result in the suspension of 20 times the standard. There is reason to believe that the result would have been the same had the suspension varied in exactly the same proportion as the serum dilution, that is to say 16 times. It is therefore justifiable to calculate the agglutinating dose for a suspension 20 times the standard from a result gained from titration of serum on the standard suspension, and *vice versa*.

Experiment 3. Combination of Antigen and Antibody at Various Hydrogen Ion Concentrations.—0.1 cc. quantities of antityphoid serum were diluted 1:30 with glycocoll-acetate-phosphate buffers of pH 7.5, 5.2, 3.9, and 3.3. 3.0 cc. of serum so diluted were then mixed with equal volume of typhoid suspension, 10

times the standard concentration. Parallel control tubes were set up with 3.0 cc., 1:30 serum + 3.0 cc. of distilled water. The eight tubes were incubated at 43°C. for 2 hours, centrifuged at 3,000 R.P.M. for 30 minutes, and the supernatant fluids diluted to 0.001 cc. (1:1,000) with buffer pH 6.0. This brought

TABLE III.

Combination of Antigen and Antibody at Various Hydrogen Ion Concentrations.

[pH at which absorption took place.	Supernatant fluids after absorption. Concentration in buffer pH 6.0 (cc. × 10^{-4}).							Agglutinating doses remaining.*	Agglutinating doses absorbed.
	100	50	25	12	6	3	1.5		
pH 7.5 Absorbed with 10 × *T* suspension.	++	−	−	−	−	−	−	<3	>50
pH 7.5 Control.	C.	C.	C.	C.	C.	++	++	55	
pH 5.2 Absorbed with 10 × *T* suspension.	++	+	−	−	−	−	−	<3	>50
pH 5.2 Control.	C.	C.	C.	C.	C.	++	++	55	
pH 3.9 Absorbed with 10 × *T* suspension.	C.	C.	C.	++	+	Tr.	−	14	41
pH 3.9 Control.	C.	C.	C.	C.	C.	++	+	55	
pH 3.3 Absorbed with 10 × *T* suspension.	C.	C.	C.	C.	++	Tr.	−	27	27
pH 3.3 Control.	C.	C.	C.	C.	C.	++	+	55	

* Agglutinating doses calculated for three volumes of 10 times standard suspension, since this was the concentration used in the absorbing suspension.

all supernatant fluids to a common pH. These neutralized diluted supernatant fluids were now diluted with pH 6.0 buffer by 2's to 0.000015 cc. and 1 cc. of standard suspension added to each tube. Incubation for 14 hours at 43°C. The result is given in Table III.

The experiment summarized in Table III shows that at pH 3.9 and more especially at pH 3.3, much less immune body is absorbed than at pH 5.2 and 7.5. There is no sensible difference between the amount absorbed at the latter two hydrogen ion concentrations. In both cases at least 94 per cent of the agglutinating substance was removed. The removal, however, was not complete in any case. Now, the agglutinating power of immune serum is distinctly higher at pH 5.2 than at pH 7.5 (1, 3). Therefore, it is evident that this increased effectiveness cannot be ascribed to a more complete quantitative union at 5.2 than at 7.5.

This experiment was subsequently elaborated and modified in certain respects. In the first place it is desirable, for constant results, to use a more concentrated absorbing suspension. Again the reaction is better conducted at room temperature than at 43°C., since at lower pH (3.3 to 3.0) the destructive effect of temperature on immune body begins to be a marked one. Finally, it seemed wise to conduct the experiment at a greater number of C_H, thus filling in the gaps existing in Experiment 3.

Experiment 4. Combination of Antigen and Antibody at Various Hydrogen Ion Concentrations.—2.0 cc. quantities of antityphoid horse serum, diluted 1:50 in buffer mixtures, pH 7.5, 5.2, 4.7, 3.9, 3.3, 3.0, and 2.7 were mixed with 2.0 cc. amounts of *B. typhosus* suspension 20 times the concentration of the standard (organisms from 150 cc. broth suspended in 8 cc. distilled water). Similar amounts of immune serum diluted with buffers of same pH were mixed with equal amounts of distilled water. These latter served as controls. All tubes were allowed to stand at room temperature (18°C.) for 11 hours.

The tubes with the immune serum *B. typhosus* mixtures were then centrifuged at 3,000 R.P.M. for 30 minutes. The supernatant fluids, together with the controls (immune serum + distilled water) were now diluted 10 times with buffer pH 6.5, bringing all mixtures to that pH.

These dilutions, equivalent to 0.001 cc. of original serum, were diluted by 2's to 0.000007 cc. of the original; 1 cc. of standard 4 times washed distilled water suspension was added to each tube, and all were incubated at 43°C. for 14 hours.

The smallest amount causing complete agglutination was taken as the criterion for the agglutinating unit. The agglutinating units recorded after the last incubation (supernatant fluids + standard suspension) were calculated to the agglutinating units required for the absorbing suspension (20 times the standard). This was done by dividing by 40 (since twice the volume of suspension was used in the adsorption experiment), in accordance with Experiment 2.

The results are given in Table IV.

TABLE IV.

Combination of Antigen and Antibody at Various Hydrogen Ion Concentrations.

pH	Tube.	Mixture.	Total agglutinating units present.*	Units remaining after 11 hrs. at 18° C.	Units absorbed.
7.5	1	Antityphoid serum absorbed with concentrated suspension.	16	< 1.3	>14.7
	2	Antityphoid serum + distilled water. Control.	16	16.0	0
5.2	3	Antityphoid serum absorbed with concentrated suspension.	16	< 1.3	>14.7
	4	Antityphoid serum + distilled water. Control.	16	16.0	0
4.7	5	Antityphoid serum absorbed with concentrated suspension.	16	< 1.3	>14.7
	6	Antityphoid serum + distilled water. Control.	16	16.0	0
3.9	7	Antityphoid serum absorbed with concentrated suspension.	16	1.3	14.7
	8	Antityphoid serum + distilled water. Control.	16	16.0	0
3.3	9	Antityphoid serum absorbed with concentrated suspension.	16	2.0	14.0
	10	Antityphoid serum + distilled water. Control.	16	16.0	0
3.0	11	Antityphoid serum absorbed with concentrated suspension.	16	8.2	7.8
	12	Antityphoid serum + distilled water. Control.	16	16.0	0
2.7	13	Antityphoid serum absorbed with concentrated suspension.	16	8.2	7.8
	14	Antityphoid serum + distilled water. Control.	16	16.0	0

* Calculated for 2 cc. of 20 times the standard suspension.

Control experiments on the effect of time showed that the combination was almost instantaneous, in agreement with results on the potential (1). The results therefore are not due merely to a different speed of combination but to a difference in the final stage.

Table IV confirms the conclusion drawn from Experiment 3; *i.e.*, that there is no demonstrable difference between the amount of immune body absorbed at pH 4.7 and that taken up at 5.2 and 7.5.

Relation of the Results of the Absorption and Agglutination Experiments.

The experiments described in the preceding paper (1) show that the amount of immune serum or of protein required to agglutinate bacteria depends entirely on the pH at which the experiment is performed. The nearer the pH is to the acid agglutination zone of the organism in question, the smaller the amount of serum required to agglutinate. This result has also been obtained by Michaelis and Davidsohn (3) and by Eggerth and Bellows (4). The present experiments show that this result cannot be ascribed to an increase in the amount of antibody combined in this region nor to any increase in the amount of immune body. These results also show that the combination between organism and immune body is independent of the charge, since the amount of immune body combined at pH 4 to 5, where the organism is negative and the immune body positive, is the same as in the range between 5 and 8, where both are negative. The influence of salt on the combination of antibody and organism also contradicts the idea that the combination is caused by opposite electric charges. If this were the case the combination should be greatest when the charges were greatest. Salt, however, decreases the charge but increases the combination. The same fact is brought out by Loeb's (7) experiments with collodion treated with gelatin. The collodion is always negative yet will become coated with alkaline gelatin solutions in which the gelatin is also negative.

The present experiments could be explained by assuming that the immune body was always *positive* and that the reversal found by Michaelis (and which was confirmed in the course of this work) was due to the fact that the immune body was carried along with the globulins as is the case with pepsin (Pekelharing and Ringer (5)). This explanation, however, could not account for the experiment shown in Fig. 3 of the preceding paper (1), which shows that at a pH of 3.0, where the organisms themselves are positive, the addition of serum *increases* this positive charge. It would seem to be improbable

that an increase in a positive charge could be caused by combination with a substance having a negative charge.

The experiments may be simply accounted for if it be supposed that the antibody or protein forms a film on the surface of the organism and is held by the ordinary forces of valence. This mechanism has been found by Langmuir (6) to exist in the case of oil films on water. The portion of the surface that is covered would then acquire the characteristics of the added substance. It is evident from this point of view that the smaller the charge on the original particle, the smaller the amount of the surface which would have to be covered in order for the particle to have the charge of the added substance; in other words, the less the amount of substance necessary to agglutinate. This is the experimental fact. This is clearly shown in Fig. 1 in which the charge on the organisms, the amount of serum to agglutinate, and the amount of antibody combined, are plotted against the pH. The figure shows that there is no relation between the amount of antibody in combination and the amount required to agglutinate, but a very close connection between the amount required to agglutinate and the charge carried by the organism.

The independence of the agglutination and sign of charge is also shown in Table V. In this experiment the amount of serum necessary to agglutinate organisms in NaCl in which they are negative is compared to the amount of serum required to agglutinate the same suspension in Cu acetate in which they carry an equal positive charge.

The mechanism of film formation will also account for the results of Arkwright (8) who found that *Bacterium coli*, sensitized with washings from *Bacillus typhosus*, could be agglutinated by anti-*Bacillus typhosus* serum. It was found that the same experiment could be performed with egg albumin. *Bacillus typhosus*, in the presence of egg albumin, could be agglutinated with anti-egg albumin serum.

These experiments show, therefore, that the combination between organism and agglutinin (or protein) is not *due to* opposite electric charges, but that the effect on the charge is the *result* of the combination.

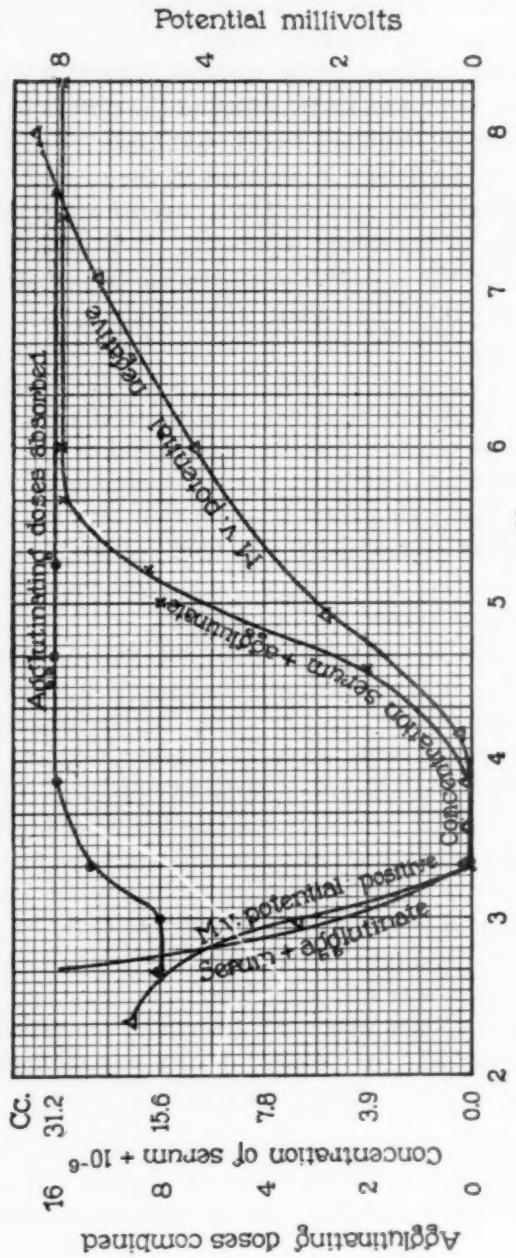
Potential millivolts
8
4
2
0
pH
2
3
4
5
6
7
8
Agglutinating doses combined
16
8
4
2
0
Concentration of serum + 10⁻⁶
Cc.
31.2
15.6
7.8
3.9
0.0
Agglutinin of doses absorbed
M.V. potential positive
Serum + agglutinate
Serum + agglutinate + undue concentration
Coagulation
M.V. potential negative

FIG. 1.

TABLE V.

Precipitation with Immune Serum in the Presence of Copper Acetate or Sodium Chloride.

20 cc. one-half suspension + 40 cc. copper acetate or sodium chloride of concentration noted.

2 cc. resulting suspension + 0.5 cc. antityphosus rabbit serum.

Final concentration of salt.	Copper acetate 0.10 N.	Sodium chloride 0.02 N.
Migration velocity under potential gradient of 1 volt per cm., μ *per sec.*	+0.50	−0.56
Potential millivolts.	+6.5	−7.0
Concentration of immune serum.	Agglutination after 20 hrs.	
1:4 } Immediate precipitation of copper	C.	C.
1:12 } hydroxide and serum protein.	C.	C.
1:36 }	C.	C.
1:108	C.	C.
1:324	C.	C.
1:976	+++	C.
1:2,700	+++	C.
1:8,100	++	+++
1:2,500	++	+++
1:7,500	++	+
1:22,000	+	+
0	±	−

SUMMARY.

1. The amount of immune body required to agglutinate a suspension of *Bacillus typhosus* increases in direct proportion to the concentration of the suspension.

2. The amount of immune body combined with the organisms is constant from pH 9 to pH 3.7. Below the latter value the amount in combination is decreased.

3. The addition of immune serum to a suspension of *Bacillus typhosus* at a pH of 2.5 increases the positive charge of the organisms.

These results are contradictory to the idea that the combination is caused by a difference in the sign of the charge carried by the immune body and the organism. They agree with the assumption that the immune body forms a film on the surface of the organism and that the effect on the charge is the result of this film.

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THE STABILITY OF BACTERIAL SUSPENSIONS.

V. The Removal of Antibody from Sensitized Organisms.

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The experiments described in the preceding paper (1) show that the combination of antibody and organism in the case of *Bacillus typhosus* is less on the acid side of pH 4.0 than on the alkaline side. It seemed possible, therefore, that antibody could be more completely removed by washing at pH 3.0. Experiment showed, however, that this was not the case. It was further found that much more antibody could be recovered by washing with distilled water than by washing with salt solution. This is in confirmation of the results of Huntoon (2) and other workers. The result of such an experiment is given in Table I. The technique was the same as that described in the preceding paper. It is evident that some of the immune body is easily removed but that the equivalent of about twelve agglutinating doses remains on the organisms. The suspension which had originally less than this number did not give up any measurable immune body. If it be supposed that the immune body forms a film on the surface of the organism, this is the result that would be expected. It has been shown by Langmuir (3) in the case of gaseous films that the first layer of molecules can be removed only with the greatest difficulty, whereas the succeeding ones are relatively easily removed. It was shown in a former paper that with optimum salt concentration agglutination occurred with one-eighth to one-sixteenth of the amount of serum required to cause agglutination in the most dilute salt. This result agrees with the present experiment in indicating that agglutination will occur when the surface is about one-eighth covered and therefore that a complete surface film represents about eight agglutinating doses.

TABLE I.

Recovery of Immune Body from Bacterial Sediments.

Supernatant fluid.	pH at which sediment had absorbed immune body.	Supernatant fluid diluted with buffer pH 6.3, 43°C., 14 hrs.						Units in sediment before washing.	Recovered.	Antibody remaining on organism.
		0.5	0.25	0.125	0.06	0.03	0.015			
Supernatant fluid No. 1 from buffer pH 7.5 treatment.	7.5	++	Tr.	−	−	−	−	16	−	16
	5.2	+	−	−	−	−	−	16	−	16
	4.7	+	Tr.	−	−	−	−	16	−	16
	3.9	+	Tr.	−	−	−	−	14.7	−	15
	3.3	++	Tr.	−	−	−	−	14	−	14
	3.0	++	Tr.	−	−	−	−	7.8	−	7.8
Supernatant fluid No. 2 from distilled water treatment.	7.5	C.	C.	C.	C.	++	Tr.	16	4.1	12
	5.2	C.	C.	C.	C.	++	+	16	4.1	12
	4.7	C.	C.	C.	C.	++	Tr.	16	4.1	12
	3.9	C.	C.	C.	++	++	Tr.	16	4.1	12
	3.3	C.	C.	++	+	−	−	14	1.7	12.3
	3.0	Tr.	−	−	−	−	−	8.2	<1	7.8

TABLE II.

Effect of Repeated Distilled Water Washing on the Acid Agglutination Optimum of B. typhosus Laden with Immune Body.

Series.	No. of washings of sediment + immune body.	43°C. 14 hrs.								
		7.9	7.5	6.6	6.0	5.5	5.0	4.4	4.0	3.3 = pH of buffer.
1	Not washed.	++	++	C.	C.	C.	C.	C.	C.	C.
2	1	+	+	+	++	++	C.	C.	C.	C.
3	2	Tr.	+	+	++	++	C.	C.	C.	C.
4	3	Tr.	Tr.	Tr.	+	+	++	C.	C.	C.
5	4	−	Tr.	Tr.	+	+	++	C.	C.	C.
6	5	−	Tr.	Tr.	+	+	++	C.	C.	C.
7	6	−	−	Tr.	+	+	++	C.	C.	C.
8	7	−	−	Tr.	Tr.	+	+	C.	C.	C.
9	8*	−	−	Tr.	Tr.	Tr.	+	++	C.	C.
10	Control suspension (no immune body).	−	−	Tr.	Tr.	Tr.	Tr.	+	C.	C.

* Stood over night at room temperature in distilled water, 3.0 cc.

The question arises whether all the immune body can be removed if washing is sufficiently prolonged. This cannot be determined by testing for the recovered antibody, since the solution is so dilute that no reaction is obtained.

It was shown in a preceding paper (4) that the immune serum widened the acid agglutination zone and moved it to the alkaline side. The limit of acid agglutination, therefore, serves as a sensitive test for the presence of immune body. The following experiment shows that sufficient washing removes all the immune body.

6 cc. serum anti-*typhosus* (1:50 in buffer pH 6.3) + 6 cc. of 10 times standard *typhosus* suspension, 18°C., 5 minutes.

The mixture was centrifuged, the supernatant fluid pipetted off, and the sediment resuspended in 10 cc. of distilled water.

1 cc. of the resulting heavy suspension was diluted to 10 cc. with distilled water, and of this second suspension 1 cc. amounts were tested against buffer series pH 7.9 to 3.3.

The balance of the heavy suspension (9 cc.) was centrifuged, resuspended in *9 cc.* of distilled water, 1 cc. removed, diluted to 9.0 in distilled water, as before, and the resulting suspension tested against the same buffer series.

This process was repeated 8 times, the distilled water for washing being reduced in amount by 1 cc. each time. This was done to keep the concentration of the original suspension of organisms constant, since 1 cc. was removed at every washing for the preparation of the dilute suspension to be tested against the buffer series.

Table II shows that the acid agglutination zone of organisms laden with immune body is greatly broadened. Thus in Series 1, Table II, the unwashed sediment is agglutinated completely at pH 6.6, and ++ agglutination occurs at pH 7.5 and 7.9. As washing proceeds, and the immune body is removed, progressively higher C_H is necessary to cause complete flocculation. At the eighth washing (Series 9) the agglutinability by acid has returned practically to that of organisms to which no immune body has been added—Series 10. The immune body can therefore be completely removed by sufficient washing.

This experiment suggests the use of this method as a test for the presence of agglutinins in bacterial suspensions. A modification of the method would be to determine the salt concentration required for agglutination, since this is also a sensitive test for immune body.

SUMMARY.

1. The removal of antibody from *Bacillus typhosus* is no more complete at pH 3 than at pH 7.

2. Approximately twelve agglutinating doses are firmly combined with the organisms. Immune body in excess of this amount is easily removable by distilled water.

3. A method of testing for the presence of immune body on the organism is described which depends on the difference in the acid agglutination of sensitized and unsensitized organisms.

4. Repeated washing in distilled water will serve to remove all the immune body from sensitized bacteria.

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THE ADHESIVENESS OF LEUCOCYTES TO SOLID SURFACES.

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Biologists not infrequently have occasion to define living cells as *sticky*, without careful analysis of what the term implies and without attempting to measure quantitatively the degree of stickiness. Previous experiments on the phagocytosis of solid particles by leucocytes (1) have naturally led to an interest in the stickiness which is such a prominent characteristic of these cells. Experiments are reported in this paper which were intended as a quantitative treatment of *stickiness*, and also as an experimental investigation of the theoretical homology between the spreading of a cell on a small particle (phagocytosis) and the spreading of a cell on a flat surface. The latter is, at least, closely related to stickiness. In both respects the experiments have been somewhat of a disappointment, but as a venture in a rather novel direction their publication may find justification. It seems at least worth pointing out that measurable changes in the stickiness of leucocytes may be produced by small changes in temperature, concentration of salt, serum and hydrogen ions. It is hoped that further measurements of the stickiness of other cells by other investigators may yield results of perhaps greater significance.

The terms *adhesiveness* and *stickiness*, which are used rather loosely throughout this report, must be considered as defined by the technique of the experiments by which this property is measured. Thus, the stickiness varies with the method of measurement. The following summary, from a recent theoretical discussion (2) of stickiness and phagocytosis may, however, be helpful in elucidating what are believed to be the principles involved in the adhesion of a cell to a slide.

1. The force holding a cell to the slide is the force of surface tension as exerted on the cell fluid, cell solid, and solid fluid boundaries.

2. A cell may fail to stick to a slide for one of three reasons: (*a*) the angle of contact predicted from surface tension may be greater than 180° (*i.e.*, the slide is tangent to the cell); (*b*) the cell may be too rigid and spherical to present a sufficient surface of contact in spite of surface tension; or (*c*) the cell may be so fluid that it tears away even though a thin invisible film of the cell widely spread out, may continue to adhere.

3. The extent to which a cell spreads on a slide is a measure of its adhesiveness to the slide only when the consistency of the protoplasm and the force tending to detach the cell are the same throughout the series measured.

For a discussion of the differential adhesiveness of blood cells, reference is made particularly to a paper by Tait (3), who remarks upon the extraordinary complexity of the interrelationship involved between cells, fibrin, endothelium, etc. This is possibly due to the fact that the adhering materials are themselves watery and are in a watery medium so that the surface tension forces involved are more nearly equal and thus more delicately balanced than would be the case in an air medium. Our usual ideas of stickiness are very incompletely and hence inaccurately typified by glue, a watery material adhering to a dry substance in an air medium. Tait considers chiefly invertebrate blood cells as does also Loeb (4). Sawtchenko (5) and Ponder (6) have made reference to the adhesiveness of mammalian polymorphonuclear leucocytes. Wolf (7) has filled agar chambers with blood and counted the number of leucocytes remaining on or in the agar after washing out the clotted blood with warm sodium chloride solution. She interprets the results as a measure of the chemotactic effect of substances dissolved in the agar although the firmness with which they adhere to the agar would seem to be another minor factor. Barikine (8) alone has made a preliminary attempt to measure the adhesiveness of leucocytes to glass. His method was to place a drop of leucocyte suspension on a slide and leave it in a moist chamber until the cells had settled out. A fine stream of salt solution was then allowed to run over it and counts were made of the number of cells which still remained on the slide over a given area.

Since it was desired to study the adhesiveness of cells to opaque materials like coal, it became necessary to develop some method whereby the number of leucocytes adhering could be measured by counting the number which were washed off.

Measurement of Stickiness by the Stream Method.

For this purpose the cells were allowed to settle out in a vessel made by sealing (with paraffin or wax) a short cylindrical glass tube, ground flat at one end, to a microscope slide or any other smooth, flat surface to be investigated. After the cells had been resting on the bottom for an hour or more, the whole apparatus was clamped to a motor which revolved slowly (17 to 20 revolutions per minute) and was rotated end for end (*i.e.*, about a horizontal axis) usually for 15 revolutions. The open end of the vessel was closed by a rubber stopper having a capillary air vent. During the rotation of the vessel the circulating *stream* of liquid washed off a certain proportion of the cells from the bottom. This percentage was determined by removing the rubber stopper, pipetting out the liquid, and counting on a hemocytometer the number of cells present.

The cells were usually fixed with a small amount of acetic acid of appropriate strength which depended somewhat upon the solution in which the cells were suspended. It was customary to use 0.13 cc. of 5 per cent acetic acid delivered by an automatic pipette into 1.2 cc. of the cell suspension. The sizes of the tubes used varied in different experiments from 1.0 to 2.0 cm. in diameter and from 2 to 4 cm. in length. The volume of the cell suspension used was correspondingly varied, so that the height of the liquid was always approximately 0.5 cm. above the bottom.

Measurements of the speed of settling of leucocytes show that they will settle this distance in about 20 minutes, their velocity under gravity being 1.6 cm. per hour. An incubation time of 1 hour is, therefore, ample to bring all the cells into contact with the bottom. The cell suspension was made so dilute in all cases (approximately 1,000 cells per c.mm.) that there was room for all the cells in direct contact with the bottom.

The leucocytes used in these experiments were all obtained from peritoneal exudates in rats following an injection of aleuronat. After

12 to 20 hours the rat was killed and the exudate washed out with physiological salt solution containing 0.25 per cent sodium citrate. The resulting suspension was centrifuged once at low speed, decanted, and the cells resuspended in 0.9 per cent sodium chloride with citrate. Unless the cells were used within an hour this final suspension was preserved in an ice box at about 5°C. Variations from this procedure will be mentioned where they occur.

In the absence of specific statement the reaction of the solution in these experiments may be taken as neutral or slightly alkaline. The effect of variations in pH will be described in a separate paper.

The Adhesiveness of Leucocytes to Various Solid Materials.

The writer has shown in experiments on phagocytosis that carbon is ingested more readily than quartz (1). It was, therefore, a reasonable expectation that leucocytes would also adhere to coal better than to glass. This was found to be the case in six out of seven experiments tried, as shown in Table I. In the same table the adhesiveness of leucocytes to paraffin is also compared by the same method. The results are in accord with the fact, first demonstrated by Haycraft and Carlier (9), that blood cells do not go to pieces if suspended in oil. It has also been shown by Tait and Gunn (10) that phagocytic cells of *Astacus* blood do not ingest pure oily globules unless some emulsifying agent has been used in their preparation. It is, perhaps, rather surprising that there is not more difference between paraffin and glass in these experiments.

The fact that leucocytes do not stick to a greasy surface as well as to a clean one may also be shown by treating the surface of coal with oil or wax. Thus, 36 per cent of the leucocytes in one suspension were found sticking to normal coal while only 22 per cent stuck to coal treated with paraffin oil. Similarly, coal polished with tripoli, a waxy material containing an abrasive, retained only 80 per cent of the leucocytes while untreated coal retained 89 per cent. There seems to be no way of proving, unfortunately, that this difference between glass and coal is not due to the greater microscopical roughness of the coal, which no amount of polishing will remove. In attempting to compare the effect of rough and smooth surfaces from this point of view, it was found that the percentage of leucocytes

sticking to ordinary glass was on an average 10 per cent less than on ground glass, the figures being 31, 38, 64, and 77 per cent as opposed to 43, 54, 70, and 85 per cent respectively. Similarly, the percentage of cells sticking to rough porcelain tile was reduced from 96 to 90 per cent by careful polishing of the surface. On smoothly polished coal, however, it was not found possible to increase the number sticking by scratching the surface with coarse sandpaper or with the point of a knife, the percentage of leucocytes sticking on these three surfaces being 67 per cent on the smoothest as compared to 63 and 54 per cent on the roughened samples. The nearest approach to a proof that roughness was not the only factor involved was an experiment

TABLE I.

Percentage of Leucocytes Which Adhere to Coal, Glass, and Paraffin.

Coal.	Glass.	Paraffin.
	77	61
28	20	
69	69	
65	30	
67		52
79	38	
89	77	
95	64	59
	39	38
67	32	18

in which 95 per cent of the leucocytes stuck to a piece of highly polished graphite, 91 per cent to rough graphite and only 70 per cent to ground glass. The ground glass was prepared by rubbing with fine alundum flour and might be regarded as rougher than the polished graphite though retaining fewer of the cells.

These results show then an apparent parallelism between phagocytosis and stickiness. When the comparison is made in acid solutions, however, the parallelism fails. As will be shown in a later paper, quartz particles are phagocytized more readily than carbon in acid solutions. No such reversal of preference is found in measurements of the stickiness of leucocytes to glass and coal in acid solutions as shown in Table II. At pH 6.05, 6.57, and 7.30 adhesion is greater

to coal or to graphite than to glass. This discrepancy between theory and fact is one which cannot be explained by changes in the rigidity of the leucocytes due to the acidity, for such changes would affect their reaction to both quartz and carbon equally. Three possible explanations are: (*a*) that the adhesiveness to carbon is maintained in acid solutions by the greater roughness of the carbon; or (*b*) that the samples of carbon used for particles and for flat surfaces did not behave alike toward H ions. The carbon used for phagocytosis was prepared from coal purified by superheated steam and washed a great

TABLE II.

Percentage of Leucocytes Sticking to Carbon and Glass When the pH Varies.

pH	Coal.	Glass.	Graphite.	Glass.
	per cent	*per cent*	*per cent*	*per cent*
7.30	73	68	91	67
	81	65	60	65
6.57	64	41	82	44
	70	59	46	52
6.05	55	0	18	0
	55	0		
	65	0		

Mixtures.—10 cc. leucocytes suspended in NaCl 0.9 per cent plus 1 cc. serum, plus 1 cc. M/20 phosphate buffer solution. Figures strictly comparable only in pairs, glass *versus* coal, graphite *versus* coal at each pH. In other respects there were slight differences due to varying sizes of tubes, different rates of rotation, etc. The general increased stickiness in alkaline solutions is, however, a fact.

many times in distilled water. The coal used for the experiments on stickiness was untreated except for a vigorous washing in soapy, tap, and distilled water. The third and most interesting possibility is (*c*) that the limiting factor in phagocytosis is *not* surface tension. Phagocytosis seems more likely to involve other factors than does a comparatively simple, abnormal, suicidal reaction like the spreading and adhesion of a cell on a glass surface.

The results of three other experiments in which the adhesiveness of leucocytes to various other surfaces was compared are shown in Table III. The differences found were not very great and can possibly be accounted for by slight amounts of the metals which may have

gone into solution or by films of grease which may have been present in spite of all attempts to avoid them. The difficulty in interpreting these results led to an abandonment of plans for extending and repeating the observations.

TABLE III.

*Percentage of Leucocytes Adhering to Various Surfaces.**

Experiment A.		Experiment B.		Experiment C.	
Nature of surface.	Stuck.	Nature of surface.	Stuck.	Nature of surface.	Stuck.
	per cent		*per cent*		*per cent*
Fused quartz.	88 ± 0.42	Sulfur.	52	Zinc.	83, 73
Glass.	86 ± 0.35	Nickel.	49	Nickel.	72
Nickel.	84 ± 0.75	Tungsten.	48	Glass.	69
Ivory.	83 ± 0.64	Zinc.	49, 40, 41	Quartz.	67
Mica.	79 ± 0.92	Tin.	46, 36	Aluminum.	67
		Aluminum.	27, 38	Tungsten.	65
				Tin.	65, 61
				Sulfur.	43

* In general the surfaces used were washed in hot soapy water, hot tap water, and finally distilled water, and allowed to dry. The fact that water spread evenly over all the surfaces with the exception of sulfur was taken as evidence of the absence of a film of grease. Before washing, the surfaces were made as smooth as possible by polishing with emery paper, carborundum, and, finally calcined alundum flour. For tin and zinc surfaces, molten samples of the pure metal were poured into moulds. The sulfur surface was similarly prepared by melting pure crystals. We are indebted to Dr. W. T. Bovie for many of the surfaces used. In Experiments A and B the cells were suspended in 0.9 per cent sodium chloride plus a phosphate buffer solution (M/60) to give a pH of 7.5, but the phosphate was replaced by 12 per cent serum in Experiment C. This may account for the different order obtained in the last experiment. The probable error of the figures as far as the counts themselves go is given in Experiment A, and may be regarded as typical of all the experiments made by this method. Since the error is always an approximately constant percentage of the number of cells washed off, small percentages of cells left sticking to the slide are determined with proportionately greater error.

Serum.

If the spreading and sticking of leucocytes on glass or other materials upon which they are allowed to settle is rightly regarded as compar-

able to the phagocytosis of solid particles, the addition of serum to a suspension of leucocytes in 0.9 per cent sodium chloride should increase the number adhering to the bottom just as it increases phagocytosis. Indeed, Barikine (8) states that certain kinds of immune sera do increase the adhesiveness of leucocytes, and this experiment

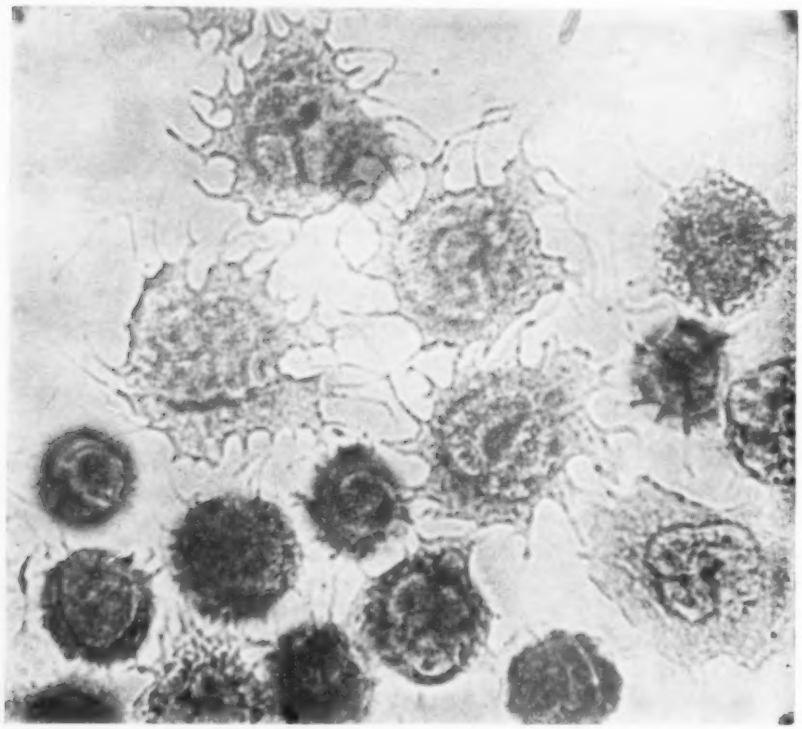

FIG. 1. Photograph of leucocytes after being in contact with the glass microscope slide for 1 hour in the absence of serum. The fine processes on the cells do not appear until they are stained with iodine. The smaller dark cells are leucocytes which have failed to spread though adhering to the slide.

has been quoted by Loeb (4) as evidence that the adhesiveness of cells plays a rôle in phagocytosis. There is no doubt that the normal effect of serum is to protect the leucocytes from spreading. If identical cells with and without serum (10 per cent or more) be examined under a microscope the spreading out of the cells deprived of serum

can be distinctly observed within a few minutes. This spreading is very evident in the photograph (Fig. 1). The normal size of the leucocytes is shown by the smaller darker cells.

In Fig. 2 are plotted the results of five experiments in which the concentration of serum was varied from 4 to 60 parts of serum in 100 parts of the mixture. The surfaces used were of glass, coal, and freshly exposed mica. All agree in showing a minimum adhesiveness

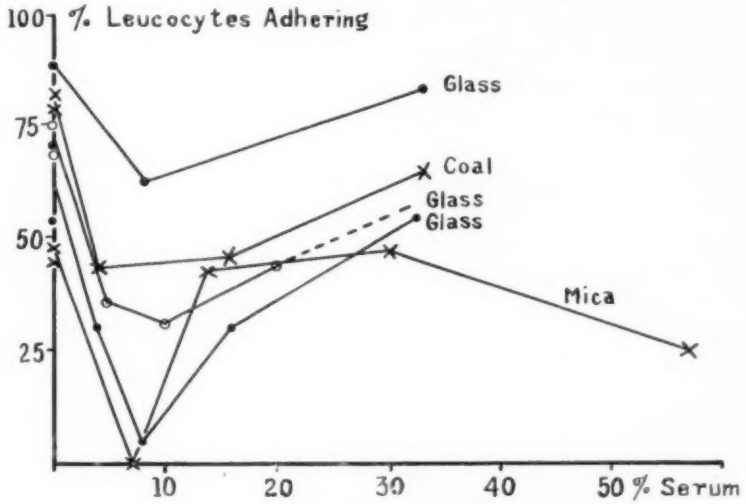

FIG. 2. Curves showing the decrease in adhesiveness of leucocytes in small concentrations of serum. Abscissæ represent the concentration of serum in per cent of normal concentration. Ordinates were obtained by subtracting from 100 per cent the number of leucocytes washed off from the coal, glass, or mica surface expressed in per cent of the total number originally present. The experiments are not comparable with one another. All were incubated at 37° C. for 1 to 1.3 hours while the leucocytes were settling on the glass.

at about 8 per cent of serum followed by a gradual increase as the amount of serum is further increased. In all concentrations of serum more cells could be washed off by rotating the vessel than in the control.

A control experiment in which the same mixtures were rotated for 1 hour in $\frac{1}{4}$ inch test-tubes at about 10 revolutions per minute at 35°C., then fixed with acetic acid, and counted, showed no significant varia-

tion except that due to a slight clumping in higher concentrations, thus decreasing the count.

In one of these five experiments (third curve from top, Fig. 2) counts were made of the number of cells visible on the slide after rotating the vessel. This was facilitated by fixing the cells with iodine. The percentage of cells stuck on the slide could then be estimated directly from the diameter of the tube, the total volume of cell suspension contained in it, the area on the slide covered by the cells counted, and the concentration of cells in the original suspension as determined on the hemocytometer. This procedure was always followed in the later experiments as it gave valuable confirmation of the results obtained by counting the cells washed off and served to

TABLE IV.

Percentage of Cells.

Serum.	Washed off.	On slide.	Lost.
per cent			
0	25	46	29
0	32	46	22
5	65	20	15
10	68	28	4
20	56	32	12

detect any loss of cells, presumably by disintegration, during the experiment.[1]

The figures obtained in this experiment are given in detail in Table IV to illustrate the method. The last column shows the percentage of cells which could not be accounted for, either because they had disintegrated beyond recognition on the glass, or because of the experimental error involved in counting. This error, due to the somewhat uneven distribution of the cells on the slide, is so large that no significance can be attributed to these figures (last column) except in the absence of serum where the 29 and 22 per cent loss indicates a

[1] Where the percentage of cells adhering is calculated merely from counts of those washed off, disintegrated cells are classed necessarily as if they were stuck to the slide. This is justified however because the disintegration is to be regarded as the end-result of the sticking and spreading on the slide.

certain amount of cellular disintegration, which is prevented by the presence of serum.

The effect of serum on the adhesiveness of cells to paraffin (see Fig. 3) at 35°C. appears to differ from that just described for glass, coal, and mica. In three experiments serum had no effect while in a fourth it caused a decrease, which, however, reached a minimum at 30 per cent instead of 8 per cent. This possibly indicates that the spreading of cells on paraffin was so much less than on glass that

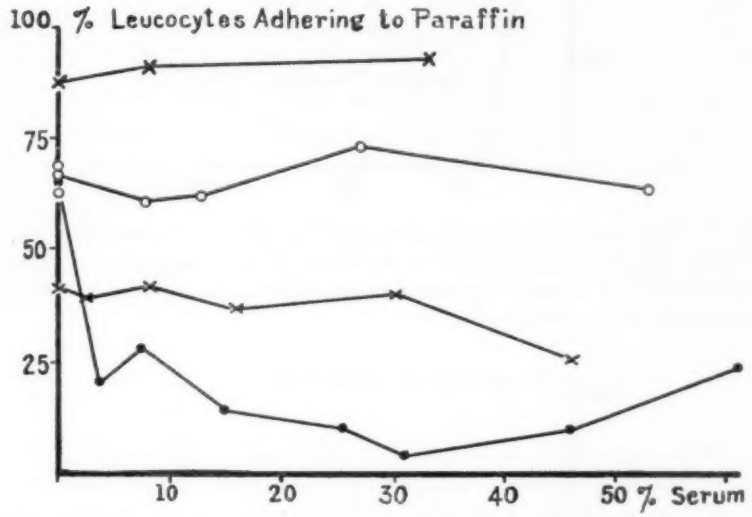

FIG. 3. The effect of increasing concentrations of serum on the adhesiveness of leucocytes to paraffin after 1 hour at 35°C. In only one case is there any significant decrease in adhesiveness. In all cases the effect differed somewhat from that on glass.

the preservative effect of serum was masked. It may also be significant that the characteristics of the two curves were reversed by varying the temperature as shown in Fig. 4. On glass at 20°C. serum is without effect though it causes a sharp decrease in adhesiveness to paraffin at 47°C.

Heating the serum to which the leucocytes are exposed decreases their adhesiveness as shown by the two comparable curves plotted in Fig. 5. This same fact is illustrated by the figures in Table V.

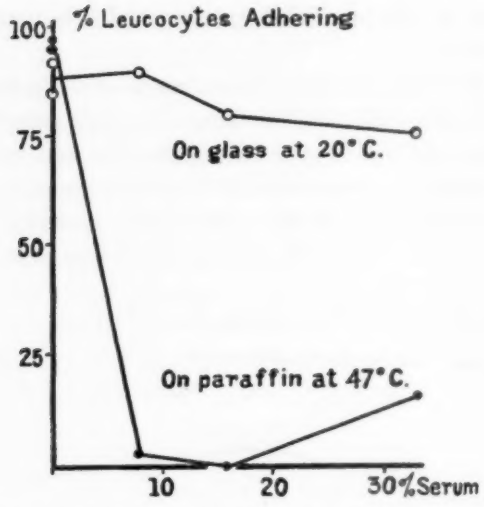

FIG. 4. Curves showing that the characteristic difference between the effect of increasing concentrations of serum on the adhesiveness of leucocytes to glass and paraffin at 37°C. is reversed by measuring the adhesiveness to glass at 20°C. and the adhesiveness to paraffin at 47°C.

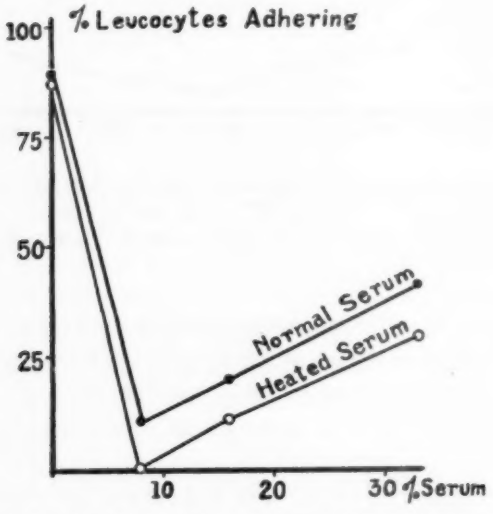

FIG. 5. Curves showing that the adhesiveness of leucocytes to glass is decreased by heating the serum to 58° for 30 minutes.

TABLE V.

Percentage of Cells.

	Leucocytes not washed off.	
	Experiment A.	Experiment B.
	per cent	*per cent*
No serum	56	74
13 per cent serum, normal	37	19
Heated to 58–60°C. for 15 min	18	5
" " 64–66° " " 10 "	15	30
After coagulating by boiling	54	55

The effect of normal serum in preventing the adhesion of cells to glass is simulated by the effect of acacia as shown in the following table where adhesion of the leucocytes is completely prevented by 0.25 per cent acacia.

Acacia.	Leucocytes stuck to glass.
per cent	*per cent*
0	59
0.03	19
0.125	25
0.25	0

Friedemann and Schönfeld (11) have observed that leucocytes washed out of the peritoneal cavity failed to spread out on the glass and continued ameboid movements only when gelatin, dextrin, egg albumin, or 2 to 8 per cent of acacia were present. They attribute this, rather absurdly it seems, to the physical resistance to spreading offered by the high viscosity of these substances.

Effect of Variations in Temperature on the Per Cent of Leucocytes Adhering.

Since the effect of serum was found to vary with the temperature, it became necessary to determine more fully the effect of different temperatures, the serum concentration remaining constant. The results of three such experiments with 0, 12, and 40 per cent serum are plotted in Fig. 6. The three experiments are not directly com-

parable as they were done with different cells on different days. The relative positions of the three curves are, nevertheless, in complete agreement with the isotherms already figured (Figs. 2, 4, and 5). The curves bring out the interesting fact that there is an optimum temperature for stickiness at 20°C. In the absence of serum the cells are comparatively insensitive to temperature, but with even a small amount of serum the maximum becomes very sharp. A similar set of curves of 0, 17, and 27 per cent serum are plotted in Fig. 7

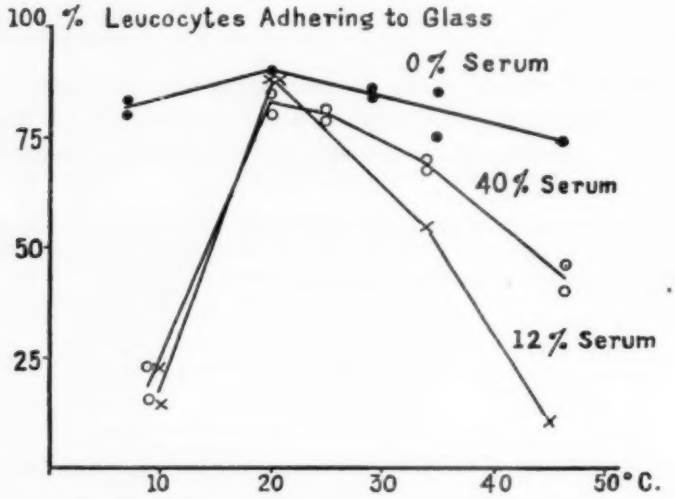

FIG. 6. Effect of varying temperature (abscissæ) on the per cent of leucocytes adhering to glass in the presence of 0, 12, and 40 per cent serum. All were allowed to settle for 1½ hours at the designated temperature before the number which could be washed off was determined. A separate vessel was used for each point.

for paraffin. Here again the curves are not comparable to each other and a comparison with the isotherms previously plotted (Fig. 3) shows that they would need some readjustment to represent the facts. There is, however, in the 17 per cent serum curve a suggestion of a broader maximum extending from 20°C. to 35°C., which would agree with the negative effect of serum at 35°C. illustrated in Fig. 3.

It seems absurd to conclude from these experiments that the stickiness of normal leucocytes in the circulation could be increased by cooling them to 20°C. Two explanations of this anomalous result

suggest themselves: (*a*) a greater fluidity of the cell at 37°C. may enable the cell to tear away from the slide more easily, leaving behind perhaps a film of protoplasm; (*b*) the maximum stickiness may be at 37°C. immediately after the cells come into contact with the glass, and at 20°C. an hour or more later, which was the usual time of measurement in these experiments.

No means of testing the first of these suggestions offers itself though it seems quite probable. Indications of the truth of the second suggestion are found in Fig. 8 where the stickiness has been measured

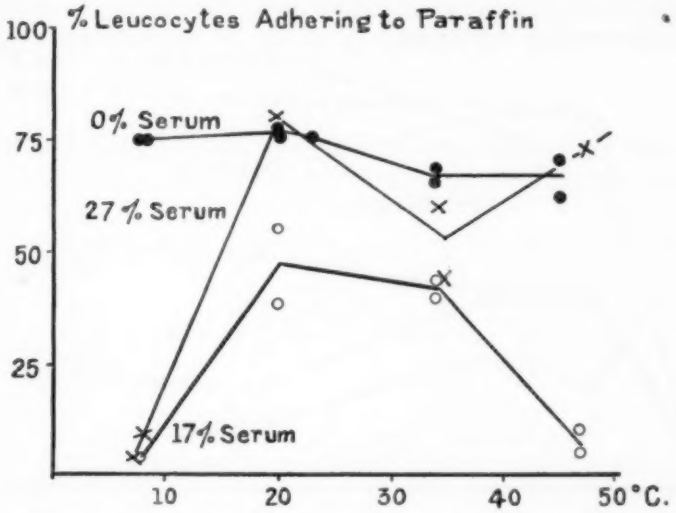

FIG. 7. Similar to Fig. 6, using paraffin instead of glass surfaces. The cells were incubated in all cases for 1¼ to 1½ hours at the designated temperature.

at various times from ½ to 6 hours. At 37° or 45°C. the stickiness evidently passes through a maximum before the end of the 2nd hour. At 3° and 20°C. the stickiness continues to increase throughout the experiment but presumably would decrease later. The temperature at which the cells would show their maximum stickiness depends, therefore, upon the length of time they remain in contact with the glass before the number which can be washed off is determined. Thus, in the upper set of curves (broken line), the maximum stickiness is at 37°C. at 1 hour, at 20°C. at 2 hours, and at 3°C. after the 3rd hour. On the whole these experiments (Fig. 8) show less change

of adhesiveness with time (6 hours) than might reasonably have been expected. If the time factor is of importance the measurements recorded in Fig. 8 after ½ hour are complicated by the fact that some of the cells have been in contact with the glass for ½ hour while others, which had to settle from the top to the bottom of the liquid in the vessel, have only just reached the slide. The following simple method was therefore devised, permitting measurements within 5 minutes after making contact. For convenience of reference it may be called the *gravity method*.

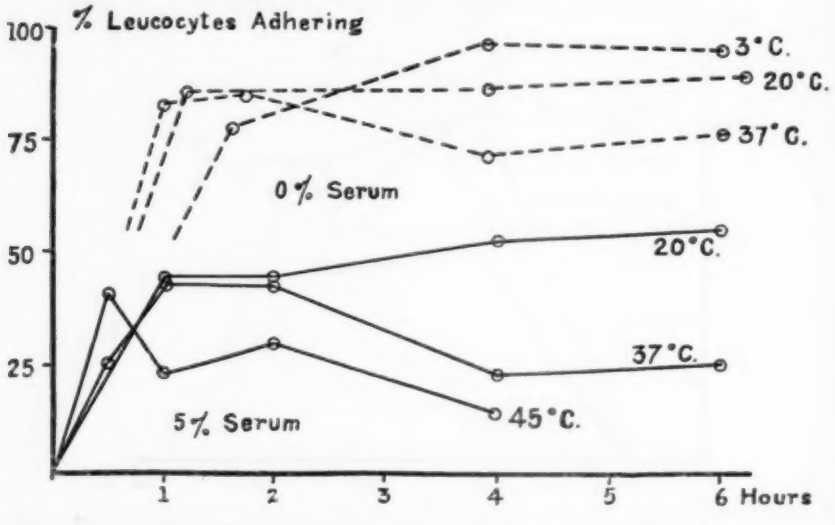

FIG. 8. Two sets of curves showing how the adhesiveness of leucocytes at different temperatures varies with the length of time they remain in contact with the glass. Typically the adhesiveness passes through a maximum which has only been reached at the higher temperatures. In the upper set of curves where no serum was present the cells were used in an M/80 solution of phosphate buffers (pH 7.4) in sodium chloride 0.9 per cent. No buffer was used in the lower set of curves.

A square cover-glass was supported on a slide on fragments of another cover-slip and was held in position by sealing it at the corners with drops of paraffin. A suspension of cells in physiological salt solution plus a given amount of serum was brought to equilibrium at the desired temperature, and a portion was allowed to run

in between the cover-slip and slide which had likewise been brought to the same temperature as the cells. Evaporation was prevented by sealing all around the cover-slip with paraffin. The adhesiveness of the cells was measured by inverting the whole preparation after sedimentation was completed (1 minute was sufficient), and counting the numbers of leucocytes on the slide and cover respectively, 1 to 5 minutes after inverting. Further change is very gradual after the first separation has been effected. By this simple and rapid method the percentage of leucocytes adhering to glass could be followed as it varied with time, beginning 5 minutes after contact occurred. The results are shown in Table VI.

TABLE VI.

Effect of Temperature on the Adhesiveness of Leucocytes to Glass.

Experiment....	A		B		C		D		E	
Time.	20°	37°	20°	37°	20°	37°	25°	37°	22°	37°
min.										
5	16	34	17	56	27	30	67	53	32	100
20–40	60	36	67	53		41*	69	55	70	100
70–80					27	13	72	54		
115–130					40					
200–300					16	25, 11	63	24		

Figures show the average per cent of cells sticking to the slide after inverting, except in E where the actual numbers of cells adhering per unit area are recorded.

* Time is 11 min.

From these figures it is evident that during the first 5 minutes of contact with the glass more leucocytes are adhering at 37° than at 20°C. (This is not true at 25°. See Experiment D.) After 20 minutes, however, this condition is usually reversed in agreement with the results obtained by the other method. In Experiments A, B, and D (Table VI) the maximum stickiness attained at any time at the lower temperature is greater than at 37°. It seems probable as already suggested, that this is due to the greater fluidity of the cell at the higher temperature. Cooling increases the rigidity of the cell and in consequence decreases both the rate with which a firm contact with the glass can be established and the rate at which the cell

will yield to some outer force, as gravity or a stream of water, tending to detach it from the slide.

The proposed explanation of the behavior of a cell can be made clearer by an example. If two postage stamps be pressed against glass slides one of which is warmed sufficiently to make the glue fluid, the warmed stamp will be found to adhere most firmly to the slide. If the pressure be continued, the cool stamp also will finally establish a contact with the slide and will then be harder to pull off than the warm stamp, though the latter would probably leave a film of glue on the slide when removed. Like the leucocytes, the stamp shows initially a greater stickiness to glass at higher temperatures, but the stickiness of the cool stamp increases with time until it surpasses that of the warm stamp which remains constant. With the stamp as with the cells, changes in consistency are of more importance than changes in interfacial surface tension. The explanation of the difference between the effects of temperature on phagocytosis of small particles and on the spreading of leucocytes on glass, is that a greater fluidity of the cell favors phagocytosis, but enables the cell to tear away from the slide more easily leaving behind probably a thin film of protoplasm, thus decreasing the *stickiness* as defined by the technique of the experiments.

All the curves in Figs. 6 and 7 showing a maximum stickiness at 20°C. were obtained during the summer of 1920 at Woods Hole. An attempt to repeat and amplify them during the following winter at Boston was at first quite unsuccessful. Finally the room temperature was raised from 22° to nearly 30°C. and all the operations of mixing the cells, centrifuging them, killing the rat, and washing out the peritoneum were carried out at summer heat although the temperatures of incubation were the same as before. The result was a perfect duplication of the previous results, 70 per cent of the cells sticking on the slide at 22°C. and only 36 and 45 per cent at 10° and 37°C., respectively. A similar though somewhat less pronounced maximum at 20°C. was obtained in another experiment by using warm sodium chloride to wash out the cells and keeping the resulting suspension at 30°C. throughout all the subsequent operations of centrifuging the cells and preparing them for the measurement of stickiness.

Just what part of the technique was affected by the temperature to this marked degree was not definitely ascertained. Attempts to relate it to the temperature at which the vessels containing the sedimented cell suspension were rotated or to the temperature at which the cells were centrifuged were not successful. Further attempts during the summer of 1921 to repeat these results were almost always successful. It never failed so long as the room was warm and the cells were washed out of the peritoneum by sodium chloride without citrate and were not centrifuged. Centrifuging them in the absence of citrate usually destroyed the maximum stickiness at 20°C. Also, experiments which were tried with cells which had stood for 6 hours more or less at room temperature or 37°C. usually failed to show the 20° maximum. A long series of such experiments gave one the impression that cells which failed to show this maximum at 20°C. had been injured in some way, as by centrifuging or by prolonged standing in the test-tube.

The following table shows the effect of centrifuging the cells with and without citrate and serum on the stickiness of the cells as measured by the stream method. The supernatant solution was not changed after centrifuging.

No.	Solution.	Leucocytes not washed off.	
		Cells not centrifuged.	Cells centrifuged.
		per cent	*per cent*
1	No serum (pH 6.8)	11–32	95
2	6.2 per cent serum (pH 7.0)	2–4	22–36
3	Sodium citrate, 0.5 per cent (pH 8.4)	7	7
	" chloride (pH 6.8)	27	

In Nos. 1 and 2 the stickiness was much increased by centrifuging. In No. 3 the presence of sodium citrate prevents this increased stickiness and decreases the stickiness of the uncentrifuged cells from 27 to 7 per cent. This last effect is doubtless due in part to the alkalinity of the citrate. In No. 1, 50 per cent of the centrifuged cells not washed off had disintegrated, while the uncentrifuged cells stuck to the slide were all perfectly normal in appearance. This shows very clearly

the injury resulting from centrifuging cells[2] without serum or citrate. All these comparisons were carried out at 20°C.

Another series of experiments was undertaken with the object of keeping the cells under continuous microscopical observation during incubation at different temperatures in order to correlate their measured stickiness with their microscopical appearance. The method consisted in sealing a cylinder of glass 1 cm. high around the small round cover-glass on the slide so that the slide could be supported just below the surface of the water in a small crystallizing dish without wetting the cover-slip. To prevent spilling, paraffin was poured on the surface of the water and allowed to harden. The glass cylinder was large enough to admit the objective of the microscope easily. The crystallizing dish was kept in a water bath or incubator at the desired temperature and could be removed for brief observation without appreciably affecting the temperature of the cells. Measurements of the diameters of such cells show that there is a tendency for them to increase at all temperatures but more rapidly at the higher temperatures. Such measurements are not very satisfactory, however, since they are complicated by disintegrative changes which alter the volume of the cell. When the stickiness would be expected to be at a maximum at 20°C. the cells are not more spread out as would be indicated by an increased diameter. In one experiment the time of the first appearance of an abnormality in the cells was carefully noted. At 47°, 37°, 27°, 20°, 15°, and 3°C. these times were 1, $2\frac{1}{2}$, 12, 24, 48, and 60 hours respectively. If the logarithms of the reciprocals of these times (*i.e.*, the logs of the rates of the disintegrative process) are plotted against the temperature, the points are found to lie roughly upon a straight line, the slope of which represents the temperature coefficient, Q_{10}, found to be approximately 3.0.

The method is necessarily not susceptible of great accuracy. The abnormal appearance which was used for an end-point in this experiment may be described as an increased refractiveness and transparency, suggesting a spreading of the cell combined with an apparent coagulation of the protoplasm giving a coarsely granular or beady appearance to the cell, a cessation of ameboid movement, and a disappearance of the normal ameboid shape of the cell.

[2] Sawtchenko in 1910 reports (5) similar injury and loss of phagocytic power after centrifuging.

These experiments confirm Loeb's (4) observation that *Limulus* cells, *in vitro*, go through the same changes at different temperatures though the rate of change varies.

Effect of Varying Concentrations of Sodium Chloride.

Hamburger (12, 13) has shown that phagocytosis of carbon particles is decreased by change in the concentration of the sodium chloride either above or below normal, 0.9 per cent. The experiments plotted in Fig. 9 show (*a*) that in the absence of serum the concentration of

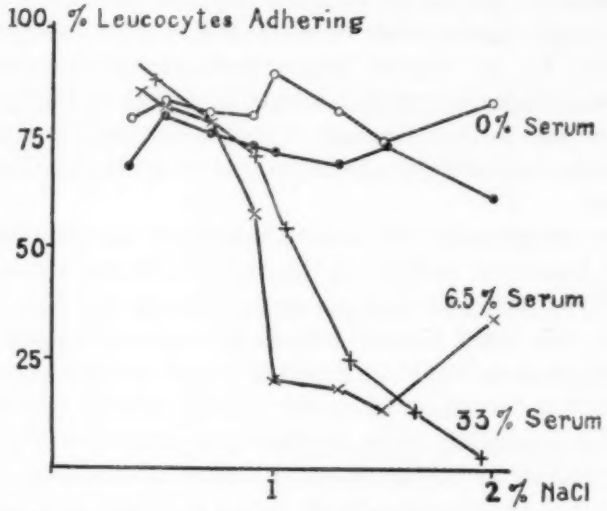

FIG. 9. Curves showing the variations in adhesiveness produced by changing the concentration of sodium chloride with and without serum. Cells were allowed to settle on the slide for 1 hour at 37°C. Stream method. Summer temperature.

sodium chloride can be changed from 0.37 per cent to 2 per cent without appreciably affecting the number of leucocytes adhering, and (*b*) that in the presence of serum any increase in sodium chloride concentration from 0.4 per cent to 2 per cent causes a pronounced decrease in the number of leucocytes sticking, there being no optimum concentration at 0.9 per cent as with phagocytosis. Otherwise stated, this means that the addition of serum causes a greater decrease in the number adhering, the higher the concentration of sodium chloride. It is significant that in this case, as with temperature, the

cells seem to be *sensitized* by the addition of serum. Furthermore the greatest sensitivity is in the neighborhood of the isotonic solution. It is instructive to compare these results with the statement of Tait and Gunn (10) that a 5 per cent solution of sodium chloride delays but does not prevent the cytolysis on glass of the amebocytes of *Astacus fluviatilis*.

Effect of Calcium Chloride, Ethyl Alcohol, and Iodoform.

Further experiments were conducted to ascertain whether the adhesiveness of leucocytes to glass could be increased by calcium chloride, ethyl alcohol, and iodoform, which were found by Hamburger (13, 14) to increase phagocytosis of carbon particles. In no case was there an increased stickiness though in high concentrations there was a slight decrease. It does not seem worth while to present these experiments in detail but the results may be summarized as follows:

In four experiments the concentration of calcium chloride was increased from 0.001 to 0.032 M, the concentration of sodium chloride being 1.7, 0.9, 0.9, and 0.62 per cent respectively. No serum was present as this would have added calcium. No significant variation in the adhesiveness could be detected except a slight decrease with increasing concentrations where the sodium chloride was hypotonic.

There is apparently, then, a discrepancy between the effect of calcium chloride on stickiness and its effect on phagocytosis (Hamburger). Too much stress should not be laid upon this result, however, since the previous history of the leucocytes could not have been identical. All the calcium chloride can hardly be removed even in the control since the repeated centrifuging which would be necessary injures the cells. That the previous treatment is important is shown by the experiments of Radsma (15) who found that calcium chloride stimulates the phagocytosis of rice-starch only when citrate or oxalate had previously been used in handling the leucocytes.

Two experiments with ethyl alcohol were tried. No significant change was produced by 0.1 per cent alcohol (6 and 8 per cent serum present), but 1 per cent alcohol decreased the number adhering from 50 to 20 per cent and again from 27 to 22 per cent.

Two experiments, with and without serum (6 per cent), with iodo-

form prepared in various concentrations up to saturation in the method described by Hamburger showed no significant variations. Hamburger (13) reports a maximum in his phagocytosis experiments within the concentration limits here used. The obvious conclusion is that the factors involved in phagocytosis and stickiness are not identical.

DISCUSSION.

In the foregoing pages an attempt has been made to test the theory that the phagocytosis of solid particles is merely a special case of the tendency of blood cells to spread upon solid objects. Dissimilarities have chiefly been found between these two processes and they may be summarized as follows:

1. In acid solutions quartz particles are phagocytized more readily than carbon particles (*vice versa* in alkaline solutions) but the stickiness of leucocytes to coal is greater than to glass in both acid and alkali.

2. Serum increases the phagocytosis of solid particles but decreases the stickiness to glass, coal, mica, and paraffin.

3. The optimum temperature for phagocytosis is at 37°C. but the stickiness is sometimes greater at 20°C. than at 37°C.

4. Hypotonic sodium chloride decreases phagocytosis (Hamburger) but increases stickiness (in the presence of serum).

5. Calcium chloride, ethyl alcohol, and iodoform, which were found by Hamburger to increase phagocytosis of carbon in small concentrations, have no effect or slightly decrease the stickiness of leucocytes to glass.

These data show that the simple conception of surface tension upon which the theory of the similarity between phagocytosis and the spreading of cells on flat surfaces is based, is not adequate to explain all the facts. There is, of course, danger in drawing conclusions from comparisons between experiments of Hamburger, for example, on phagocytosis of carbon particles, and our own experiments on the adhesiveness of cells to glass, not only because the solid surface differs in the two cases but also because the methods of obtaining and handling the leucocytes were not identical. One can hardly account in this way, however, for the opposite effects of serum, for example, on phagocytosis and adhesiveness.

Since there can be no doubt that principles of surface tension must be operative in such experiments, we have to inquire what other factors contribute to the findings. The obvious suggestion is that the effects of surface tension are obscured by changes in the internal structure or consistency of the protoplasm. This factor has recently been invoked particularly by Loeb (16) in reference to ameboid movement. The mechanism by which an increased fluidity of the cell can increase phagocytosis and decrease the stickiness as measured by our technique has already been discussed under the effects of temperature changes. It may also be suggested that an increased rigidity of the cell would be a geater factor in preventing spreading than in preventing phagocytosis because the necessary mechanical deformation is less. It seems to be just here that the surface tension hypothesis is incomplete. It is not at all certain, however, that all the discrepancies between phagocytosis and spreading, particularly the effects of serum, can be explained by consistency changes.

SUMMARY.

1. Methods are described for measuring the stickiness of cells to solid surfaces.

2. The effect of various factors such as temperature, serum concentration, sodium chloride concentration, etc., on the adhesiveness of leucocytes to solid surfaces and the phagocytosis of solid particles are compared, and certain marked differences pointed out.

3. On account of these differences it is concluded that forces of surface tension, though undoubtedly operative in determining the behavior of cells in contact with solid bodies, are not the only factors involved.

4. Ways in which changes in the structure or rigidity of the protoplasm might affect phagocytosis and adhesiveness in opposite directions are suggested.

I take pleasure in acknowledging the helpful criticism of Dr. Cecil K. Drinker in this work and the courtesies of the Marine Biological Laboratory, Woods Hole, Mass., where some of the experiments were done.

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EFFECT OF THE HYDROGEN ION CONCENTRATION ON THE PHAGOCYTOSIS AND ADHESIVENESS OF LEUCOCYTES.

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In a previous paper (1) a method has been described for measuring the adhesiveness of leucocytes to glass surfaces, and a series of results obtained by this method was reported. As a further calibration of the method it was necessary to determine what variations in adhesiveness could be produced by varying the H ion concentration. Since the method was originally devised as a means of comparing the spreading of leucocytes on flat surfaces (adhesiveness) with their spreading on surfaces of small solid particles (phagocytosis), the effect of variations in pH on phagocytosis of solid particles has also been investigated. These two series of experiments will be reported together.

This subject has been investigated qualitatively by Sawtchenko and Aristowsky (2) for leucocytes, and they found a maximum number of leucocytes sticking to their test-tubes in neutral or slightly alkaline solutions. Loeb (3) reports collaborative experiments by Blanchard indicating an optimum acidity for the migration of amebocytes of *Limulus*. In more alkaline solutions they adhere to the glass and disintegrate, while in more acid solutions their consistency is so great that they cannot move.

For a further investigation of this subject measurements of the adhesiveness of leucocytes to glass at varying pH were made by the *stream* and *gravity* methods already described. In the former method the leucocytes are allowed to settle on a glass slide forming the bottom of a glass vessel. When the vessel is rotated about a horizontal axis the resulting circulating *stream* of solution washes off a certain percentage of the leucocytes on the bottom. This percentage is determined by hemocytometer counts. In the gravity method a

slide and cover-glass preparation is inverted and counts are made of the numbers of leucocytes which drop from the slide to the cover-slip in response to *gravity*.

Results by the Stream Method.

In Fig. 1 are plotted the results of two experiments in which the stickiness of leucocytes to glass was measured in solutions of varying pH, with and without 5 per cent serum. The cells were washed out of the peritoneum of the rat with 0.9 per cent sodium chloride without

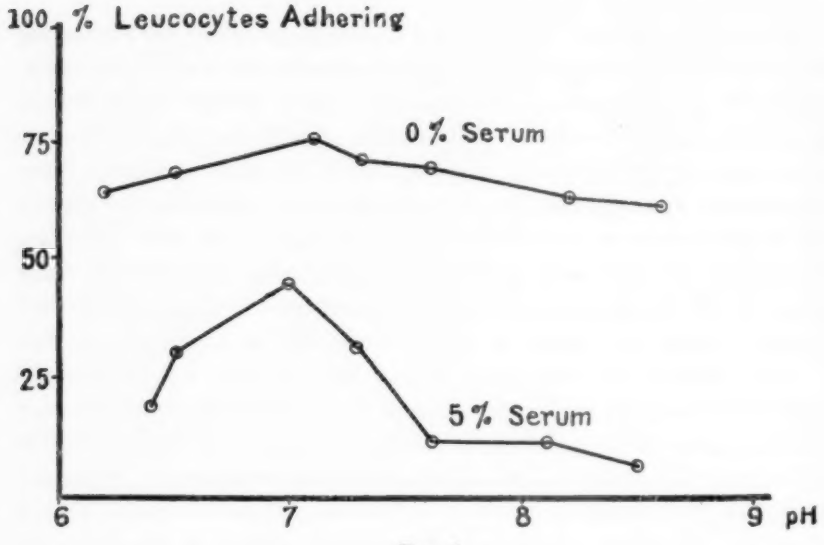

FIG. 1.

citrate at a room temperature of 25°C. They were not centrifuged but used directly. The pH was controlled by means of buffer solutions of phosphates and borates. Borates were used for the two most alkaline points in each curve. In all cases 0.3 cc. of 0.05 M buffer solutions were added to 1.5 cc. of cells or of cells plus serum. The pH of the resulting mixture was measured electrometrically. The cells were left in contact with the glass for 1 hour at 25°C., after which the vessels containing them were rotated fifteen times on a motor. Counts were made in the liquid which was pipetted off after

fixing with acetic acid, and on the slide after staining with iodine. The figures plotted in Fig. 1 were obtained by subtracting the per cent washed off from 100 per cent. The counts, made directly on the slide, which are always much less accurate, need not be given but it may be stated that they supplemented the published curves closely enough to show that there was no appreciable loss of cells during the experiment.

The significant result of this experiment is that the leucocytes show a maximum stickiness at about neutrality, and that they appear more sensitive to the variations in pH when a small amount of serum is present than they do in pure sodium chloride. Similarly it has been

TABLE I.

Effect of pH on Adhesiveness of Leucocytes to Glass.

Experiment A.				Experiment B.		Experiment C.	
pH	On slide.	Washed off.	Estimated lost.	pH	On slide.	pH	Washed off.
	per cent	*per cent*	*per cent*		*per cent*		*per cent*
5	75	15	10	6.0	1.7	6.1	43
6	72	19	9	6.4	1.9	6.5	41
7	81	17	2	7.0	9.2	6.8	50
7.6	100	11	−11	7.2	12.8	7.2	45
8	79	22	−1	7.6	4.6	7.5	44
9	53	27	20	8.0	3.4	7.7	38
10	39	19	50			8.0	43

found (1) that serum makes the cells more sensitive to variations in the temperature and to variations in sodium chloride concentration.

The results of three other confirmatory experiments are given in Table I. The protocols were as follows:

Experiment A.—Incubation time 1 hour. Washed out peritoneum with isotonic sodium chloride without citrate. Did not centrifuge. No serum added. Each figure in Table I is the average of two determinations at 20° and 37°C. respectively. The greater stickiness was found at 20°C. The cells were obviously badly disintegrated at pH 8 and 9, as the counts show. The apparent increase in the number of cells (−11 per cent lost) is due to experimental error.

Experiment B.—5.2 per cent serum. Incubation time 1 hour. Temperature 23°C. Washed out peritoneum with 0.1 per cent Na citrate in isotonic sodium chloride. Centrifuged the cells and resuspended them in isotonic sodium chloride.

Experimental error of the counts of cells washed off was greater than the per cent sticking. Hence, only the counts of cells remaining stuck to slide are given.

Experiment C.—No serum. Incubated for 1 hour at 37°C. Complete lack of significant variation is due to lack of serum and to fact that the cells were kept for 5 hours before using in isotonic sodium chloride at 37°C. Such treatment always injures cells.

By this method, then, a maximum is found varying under different conditions from pH 7.0 to 7.6, roughly. This does not altogether confirm the qualitative statement of Sawtchenko and Aristowsky (2) that leucocytes stick to test-tubes better in neutral or slightly alkaline solutions than in acid solutions.[1] Their statement is, however, confirmed by the gravity method. The discrepancies between the results by these two methods is to be explained largely by the time factor, since it is necessary to incubate the leucocytes for at least 1 hour in the *stream* method to be sure that all of them have reached the glass. The great advantage of the gravity method is that measurements can be made immediately after the cells strike the glass. Moreover, the force of gravity tending to detach the cells from the slide is much less than in the stream method, so that 90 per cent of the cells may resist gravity while only 10 per cent may resist the washing caused by rotating the vessel. This difference in itself seems sufficient to cause variations in the *stickiness* as measured by the two methods. The relative importance of cell rigidity and surface tension forces in permitting detachment of the cell may not be the same for gravity as for the force of streaming water.

Gravity Method.

The principle of this method has been described, and for the details the reader is referred to a previous paper (1). The results are summarized in Table II. 10 per cent serum was present in all preparations. It was calculated that the phosphate added left the solution very nearly isotonic, the divergence being the same for all. Irrespective of temperature it is found that the greatest stickiness is at pH 8.0. A decrease would probably occur at greater alkalinities but that is beyond the range of physiological interest.

[1] Schwyzer, also, using a special method, has concluded that NaOH increases the adhesiveness of leucocytes to glass. Schwyzer, F., *Biochem. Z.*, 1914, lxx, 447.

It may be concluded, then, that *the adhesiveness of leucocytes increases with the OH ion concentration between pH 6.0 and 8.0.* It is curious that the reverse has been found (4) true for washed erythrocytes which stick better in acid solutions when measured by the gravity method.

TABLE II.

Adhesiveness of Leucocytes to Glass in Solutions of Varying pH.

pH	No. of leucocytes sticking to inverted slide over equal areas.							
	Temperature 18°C.				Temperature 23°C.	Temperature 35°C.		
	Experiment 1.	Experiment 2.	Experiment 3.	Sum.	Experiment 4.	Experiment 5.	Experiment 6.	Sum.
6.2	13	24	1	38	37	38	14	52
6.7	(20)*	4	34	58	33	49	17	66
7.0	23	31	46	100	64	74	14	88
7.4	32	44	21	97	74	69	25	94
8.0	37	48	64	149	64	83	59	142

* Interpolated.

Areas counted were not identical in the different experiments. In Experiments 3, 4, and 6 the area is that occupied by 100 cells before inverting. In Experiments 1, 2, and 5 the area was 1 sq. mm. Solutions include 1 cc. serum, plus 1 cc. 0.05 M phosphate buffer mixture, plus 8 cc. cells in NaCl 0.9 per cent. The pH values are those of the buffers and are probably accurate to 0.1 pH. The two most alkaline solutions in Experiment 4 were pH 7.6 and 8.3 $\pm$ 0.2 respectively.

Mutual Stickiness of Leucocytes.

Having determined that the adhesiveness of leucocytes to glass was at a maximum at pH 8.0, it was of theoretical interest to determine the maximum for the mutual adhesiveness or agglutination of leucocytes. For this purpose suspensions of leucocytes in 10 per cent serum solutions of varying pH were prepared and samples of each were mounted between slide and cover-slip as if for a measurement of stickiness to glass by the gravity method. Counts were made, after 4 to 5 hours, of the number of unclumped cells. The same leucocyte suspensions were also allowed to settle out in tubes at room temperature. After 6 hours they were resuspended and the number of clumps of four or more cells were counted. Both these counts agree in

showing a greater agglutination in acid solutions (see Table III). The same conclusion was also obvious from mere inspection of the preparations.

This experiment emphasizes the fact that stickiness cannot properly be called a property of any substance or any cell taken by itself. One must state always the object to which that substance sticks and the medium in which both are suspended. Thus, in air, glue sticks to wood but not to paraffin, but in water it does not stick to wood. So when a microscopist defines a cell or tissue as *sticky* it is proper to ask, "Sticky to what?" *Leucocytes may be sticky to glass but not to other leucocytes or vice versa.* This is because stickiness depends, in part, as previously stated in a theoretical discussion of this subject

TABLE III.

Agglutination of Leucocytes.

pH	Per cent cells not in clumps on slide (4 to 5 hrs.).	No. of clumps per mm. in test-tubes (6 hrs.).
6.0	48	170
6.35	45	160
6.7	55	115
7.0	53	130
7.4	63	80
8.0	81	55
9.0	66	50

(5), on the balance between the forces of surface tension on the three interfaces concerned. A change in any one of the three phases may affect its two interfacial tensions oppositely. Particular warning should be given to avoid the idea that a *sticky* cell necessarily has the viscidity peculiar to glue. There does not seem to be any reason why a cell could not stick to a slide just as smoke particles may stick to glass without its being possible to pull the surface protoplasm out into long strands.

Effect of pH on Phagocytosis.

Two methods of measuring the phagocytosis of solid particles have been used in this laboratory, the *film* method and the *suspension* method.

Film Method.

The leucocytes were prepared as for measurements of stickiness by the gravity method. The solution is made hypertonic as to sodium chloride and enough of a suspension of quartz or carbon particles in distilled water is added to dilute it again to isotonicity. The solution is immediately allowed to run under a cover-slip pre-

TABLE IV.

Phagocytosis of Quartz Particles.

pH	Particles inside cells during 1st hr.	Particles not touching cells during 3rd hr.	No. of cells per unit area stuck to slide inverted after	
			3½ hrs.	16 hrs.
Experiment 1.	*average per cent*	*average per cent*		
6.0	6.7	70	2	1
6.7	11.7	64	49	21
7.0	16.1	43	22	7
7.3	13.7	57	131	26
7.8	6.1	75	73	8
	1st half hr.	2nd and 3rd hrs.	4 hrs.	
Experiment 2.				
6.0	4	59	45	
6.7	15	47	19	
7.0	21	29	9	
7.3	21	20	18	
7.8	12	38	55	

10 per cent serum and 0.0075 M phosphate buffer solution in final mixture. Particles about 4μ in diameter. Experiment conducted on microscope slides observed on warm stage.

pared as described for stickiness measurements by the gravity method. The particles settle out on the slide before they have time to agglutinate and are ingested by the leucocytes which are creeping about.

The results of four experiments of this sort with quartz and carbon particles are given in Tables IV and V. Phagocytosis was followed by counting: (*a*) the number of particles apparently completely inside the leucocytes over equal areas; and (*b*) the number of particles neither inside nor in contact with the leucocytes. These two counts

confirm each other satisfactorily. With quartz the optimum pH for the reaction is 7.0. With carbon the optimum is at pH 7 or 7.3 with a fairly definite tendency to fall off less rapidly than quartz in alkaline solutions. This finding will be confirmed in a later paper.

Because of interest in the theoretical relationship between phagocytosis and stickiness, the whole preparation was inverted at the close

TABLE V.

Phagocytosis of Carbon Particles.

pH	Particles inside cells during 1st half hr.	Particles not touching cells during 2nd half hr.	No. of cells per unit area stuck to slide inverted after
			1 hr.
Experiment 3.	*average per cent*	*average per cent*	
6.0	3.5	80	46
6.7	13	64	24
7.0	27	40	24
7.3	25	33	10
7.8	23	46	19
	1st ½ hr.	2nd ½ hr.	1½ hrs.
Experiment 4.			
6.0	17	62	107
6.7	22	57	152
7.0	37	29	72
7.3	35	43	291
7.8	39	49	61

10 per cent serum and 0.005 M phosphate buffer in final mixtures. Carbon suspension kept in distilled water; particles about 3μ in diameter. Experiment conducted on microscope slides observed on warm stage. Results of two different experiments are tabulated.

of each of these experiments and the percentage of cells which remained adhering to the slide were counted. The result is given opposite the phagocytosis findings. In every case the stickiness *minimum* coincides with the phagocytosis *maximum*. The significance of this fact is obscure, as is also the explanation of a minimum stickiness to glass at neutrality after 2 to 3 hours, as compared to the maximum at pH 8.0 immediately after contact. A similar result

has been obtained with the *stream* method in protracted experiments. The changes which cells undergo *in vitro* are quite different in solutions of different pH, and each of these reactions must be thought of as proceeding with its own velocity and subject to its own temperature coefficient. It is no wonder, then, that the stickiness changes with time and temperature so markedly that a large number of measurements have had to be discarded because they were apparently mutually inconsistent. All the variables could not be controlled.

The question now arises whether the apparent phagocytosis maximum at pH 7.0 is due to greater ease of ingestion or to greater availability of the particles caused by a more rapid ameboid movement of the leucocytes at neutrality. Unfortunately, the only suitable method of measuring the speed of a creeping leucocyte seems to be that of Commandon (6) who took moving pictures of them. One distrusts simple observation of the cells which appear to be more ameboid at pH 6.0 than at 7.0, and are occasionally seen elongated into a strand of protoplasm 50 to 100 microns in length. This may be due to a disturbance in the coordination of the cell, as suggested by Tait (7), such that it tries to creep in opposite directions at once, each pseudopod persistently maintaining its contact with the slide. On inverting such a preparation it is the most widely spread out and elongated cells which remain adhering to the slide. Such cells may be more spread out *in situ* but may actually migrate less and hence meet fewer particles in unit time than cells at neutrality. Cells in alkaline solutions appear more stationary than at neutrality. The more rapid clumping of cells in acid solutions might be explained by increase in ameboid movement and hence more rapid approach of cells were it not for the greater clumping in similar cells allowed to settle out and therefore already in contact. Moreover, De Haan (8), in a recent paper, reports greatest pseudopod formation in leucocytes at neutrality when they are examined in suspension by his fixation method to avoid the complications arising from contact with glass.

Suspension Method.

With such considerations in mind the effect of pH on phagocytosis of quartz in suspension was investigated, thus eliminating the rate

of cell migration as a complicating factor. Five suspensions of quartz particles and leucocytes were prepared in solutions of varying pH in small ¼ inch test-tubes at 37°C. At intervals samples were removed to a hemocytometer and the number of particles not ingested was counted. At each sampling all the tubes were inverted to prevent settling out. There was no evidence of clumping of the particles. Time curves of the reaction at each pH were plotted and from them the summary in Table VI was taken, showing the percentage of particles ingested at two representative stages in the reactions and the minutes necessary for the ingestion of 25 per cent of the particles. The results show an optimum for quartz ingestion on the acid side of neutrality, probably between 6.7 and 6.0.[2] Attention has also

TABLE VI.

Phagocytosis of Quartz in Suspensions.

pH	Per cent particles ingested after		Time for ingestion of 25 per cent of the particles (mins.).
	½ hr.	1½ hrs.	
6.0	70	76	15
6.7	79	79	5
7.0	68	70	7
7.7	41	41	24
8.1	29	41	45

10 per cent serum and 0.005 M phosphate buffer solution in final mixture. Temperature 37°C.

been called to the fact that the ingestion of carbon tended to fall off less rapidly in alkaline solutions than that of quartz. These facts will be more fully confirmed in a later paper. They indicate, however, that *the apparent phagocytosis maximum at neutrality found by the film method is due to the more rapid movement of the leucocytes* and hence the greater availability of the particles; *i.e.*, the ease of phagocytosis is not the limiting factor.

The rather striking conclusion to which these experiments lead is that *the stickiness of leucocytes to a glass slide increases with the alkalinity, while the phagocytosis of quartz particles increases*

[2] On account of the rapid agglutination of carbon particles, particularly in acid solutions, this experiment was not repeated with carbon.

with the acidity. This demonstrates very clearly that some factors other than surface tension are involved in these reactions. There seems no possibility of explaining this result by differences between the quartz and glass particles which have behaved almost identically in endosmosis experiments conducted in this laboratory.

SUMMARY.

1. Immediately after coming into contact with glass, leucocytes are most adhesive at pH 8.0 or > 8.0.

2. Agglutination of leucocytes increases with increasing H ion concentration from pH 8.0 to 6.0.

3. In phagocytosis experiments where leucocytes creep about on the slide picking up articles the optimum pH is 7.0. Here ameboid movement is probably the limiting factor.

4. The optimum for phagocytosis of quartz from suspension is on the acid side of neutrality at or near pH 6.7.

5. Phagocytosis of quartz increases with the acidity, while adhesiveness of leucocytes to glass increases with the alkalinity.

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THE INFLUENCE OF AMMONIUM SALTS ON CELL REACTION.

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The recognition of the importance of the true reaction or hydrogen ion concentration of the blood and body fluids of organisms, as opposed to their titratable acidity, has been responsible in recent years for many important advances in physiology and for a large and rapidly growing body of literature. So great has been the interest in the determination, made possible by the development of the indicator and the electrometric methods, of the pH, as such, in the case of body fluids, sea water, culture media, etc., that the fact has rather generally been overlooked that the pH of the medium to which a cell is exposed bears no necessary relation to the pH produced by that medium within the cell itself.

This is particularly true in the case of the most important of all of the various buffer systems found in organisms; namely, the carbonic acid-bicarbonate system. The author[1,2] has shown that the pH produced within a cell by a medium containing both CO_2 and bicarbonate can by no means be predicted from the pH of the medium as measured with indicators or electrometrically. Thus, cells composing the flowers of *Symphytum peregrinum*, which contain a natural indicator sensitive to weak acids, when exposed to the slightly alkaline solution resulting from the saturation of M/2 $NaHCO_3$ with CO_2, give visible evidence of a rise in intracellular acidity which is almost as rapid and striking as in the case of a strongly acid saturated solution of CO_2 in distilled water, and far more rapid than in the case of solutions of HCl whose hydrogen ion concentration is still greater.

[1] Jacobs, M. H., *Am. J. Physiol.*, 1920, li, 321; liii, 457.

[2] Jacobs, M. H., *Biol. Bull.*, 1922, xlii, 14.

Likewise, the taste receptors of the human tongue which are concerned in the sensation of sourness (*i.e.*, which are specifically stimulated by hydrogen ions) are not sensitive to most acids at a pH less acid than 3.0, but are excited even by the alkaline mixture of CO_2 and bicarbonate just mentioned.

These facts have been explained by the author as being due to a differential permeability, CO_2 being free to enter living cells while bicarbonates are not, or are to a far less extent. Consequently the equilibrium reached within the cell depends not on the mere pH of the surrounding medium but essentially on the amount of free CO_2 which the latter contains, as well as on the buffer content of the cell itself. An increase in intracellular acidity may be produced in such cases by a neutral or alkaline solution, while no change may occur with another solution, even though it shows a fairly high degree of acidity.

As far as acids are concerned, it appears that with the possible exception of H_2S, on which investigations from this point of view have been begun, carbonic acid is a unique substance owing its peculiar characteristics to the combination of two properties: (*a*) its weakness as an acid which permits at neutrality or even at low degrees of alkalinity the presence of considerable amounts of the free acid and its anhydride, CO_2; and (*b*) the extraordinary ease with which it (either as CO_2 or H_2CO_3 or both) penetrates living cells, this ease of penetration being perhaps correlated with its free solubility in lipoids.

The facts which have been mentioned naturally suggest the question: Are there any substances of alkaline nature whose behavior is analogous to that of CO_2—substances which from solutions having an acid reaction can produce intracellular alkalinity as CO_2 and from an alkaline solution can produce intracellular acidity? That there is at least one such substance is indicated by the experiments on ammonium salts which will now be described.

A solution of an ammonium salt, *e.g.* NH_4Cl, in aqueous solution on account of the weakness of NH_4OH as a base undergoes partial hydrolysis according to the equation:

$$NH_4Cl + H_2O \rightleftarrows NH_4OH + HCl.$$

Because of the greater degree of dissociation of HCl as compared with NH_4OH, the solution has an acid reaction. Now from the work of Warburg[3] and of Harvey[4] and others we know that NH_4OH (or NH_3?) penetrates living cells with the greatest readiness. HCl, on the other hand, like other mineral acids, enters with difficulty. It would appear, therefore, on theoretical grounds that in a solution of an ammonium salt, even though the reaction of the solution be acid, a cell might readily become more alkaline. That this actually occurs, at least in certain cases, may be shown in a number of ways.

During the spring of 1922 it was found that the red flowers of a hybrid *Rhododendron* cultivated in the botanical gardens of the University of Pennsylvania contain a natural indicator which is extremely favorable for showing the entrance of NH_4OH. When small portions of the blossom are placed in an N/100 or N/1,000 or even a weaker solution of this substance, the color quickly changes along the torn edges to a violet and then to a beautiful clear blue. As seen with a hand lens, or even better with the compound microscope, the change in color is extremely striking. No more favorable material could be desired for following the penetration of ammonia from solutions of ammonium salts.

Preliminary experiments *in vitro* with the expressed filtered juice of some of the flowers, using phosphate buffer solutions to regulate the pH, showed that the indicator begins to change color from red to violet at about neutrality. At pH 7.0 the change is scarcely noticeable; at pH 8.0 it is very decided, though not complete. It may be mentioned that while the first change from red to violet is the same with the juice as with the living cells, the further change with increasing alkalinity in the former case is to a dirty green rather than to a clear blue.

Having found that the indicator is unaffected on the acid side of neutrality, an M/10 solution of $(NH_4)_2SO_4$ was brought to a pH of 6.2 by the addition of a trace of free ammonia and portions of the flowers were exposed to it. In every case there was a prompt change in the color of the cells, beginning at the cut and torn edges, to a

[3] Warburg, O., *Z. physiol. Chem.*, 1910, lxvi, 305.
[4] Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507.

bright blue. Control experiments in which water alone or an M/10 solution of NaCl were used gave no such result. Also the ammonium sulfate solution when added to the extracted indicator caused no change in its red color. Finally, it was found that cells which had been sufficiently injured by heat or by a short exposure to water saturated with ether failed to turn blue. Evidently there is produced in the living cells an effect which is most reasonably explained by a differential penetration of NH_4OH from a solution in which it is present as one constituent, even though the reaction of the solution itself is on the acid side of neutrality.

The effect of pure NH_4Cl and $(NH_4)_2SO_4$ (without the addition of NH_4OH) was next tried. Solutions of both substances are considerably more acid than the one employed in the preceding experiment, and the free NH_4OH present is much less in amount. Nevertheless, distinct, even if less striking evidence than before was obtained of an increase in the alkalinity of the cells. The color change in some cases went only as far as a blue-violet, though in others a pure blue was obtained. The differences appeared to depend to some extent on the condition of the flowers, the least striking results being obtained with those which were old and somewhat wilted.

It would be expected that ammonium acetate, which is much more strongly hydrolyzed in aqueous solutions than either the sulfate or the chloride, would be more effective than they, and such is the case. Solutions of ammonium acetate of strengths varying between M/10 and M/2 to which no NH_4OH had been added were found to be as effective as the first solution used which was a mixture of the sulfate and the hydroxide. It may be mentioned that since the dissociation constants for acetic acid and for ammonium hydroxide are nearly equal, a solution of ammonium acetate has a reaction which is approximately neutral.

The results obtained with *Rhododendron* blossoms may be duplicated with animal cells. One simple, and at the same time striking experiment may be described which brings out not only the peculiar effect of ammonium salts but that of the CO_2-bicarbonate system as well.

Unfertilized starfish eggs were lightly stained in neutral red, concentrated by means of a centrifuge, and introduced with practically

no sea water into three Syracuse watch-glasses containing respectively the following solutions: (*a*) M/2 NaCl brought to pH 7.2 by means of a trace of $NaHCO_3$; (*b*) M/2 $NaHCO_3$ brought to the same pH by saturating with CO_2; and (*c*) M/2 NH_4Cl brought to the same pH by means of NH_4OH. The three solutions were identical in hydrogen ion concentration and in salt concentration, and according to the views tacitly accepted by many physiologists might have been expected to have the same effect on the intracellular reaction. Such was not the case, however. When the watch-glasses were gently agitated so as to collect the eggs in masses and these were viewed with the naked eye against a black background, the following appearances were clearly visible. The eggs in (*a*) had not changed their color; those in (*b*) had become bright pink, indicating an increased acidity; and those in (*c*) had become yellow, indicating an increased alkalinity. These effects were apparent within a few seconds; after standing for 15 minutes, as the cells began to be injured by the solutions (as shown by microscopic examination), the pink color of (*b*) and the yellow color of (*a*) began to be changed in the reverse directions until finally the three lots of eggs were practically indistinguishable in appearance.

That even a pure solution of M/2 NH_4Cl may cause increased intracellular alkalinity in starfish eggs may also be shown by the same method, though the color changes in this case are not so striking as those just described. Instead of becoming yellow in color, the eggs merely become orange-red. However, the difference between the treated eggs and the controls, when both are viewed in masses against a black background, is unmistakable.

Similar results may be obtained with animal material of a different sort. The author has found it convenient in certain studies, as yet unpublished, on the relative rates of penetration of various acids into living cells, to employ "artificial cells" which are constructed as follows: A piece of glass tubing 5 cm. long and 1 cm. in diameter is provided at one end with a lip. Over this end is gently stretched a piece of frog's skin, which is held in place with a rubber band. Within the "cell" is placed a solution of any desired buffer content and containing any desired indicator. Penetration of the acid may then be followed by changes in the color of the solution. Because the skin

of the frog is provided with glands and because there is a possibility of penetration between the cells rather than through them, the results obtained with this material would be expected to be more complicated than those secured where single cells are concerned; it appears, however, from a variety of experiments which need not here be detailed that the general behavior of the two kinds of material is in most respects surprisingly similar.

When "artificial cells," in which the "cell membrane" is composed of frog's skin are used with ammonium salts, very striking results may be obtained. With the skin turned *inside out*, an internal solution containing phenol red and neutral in reaction at the start may easily be carried beyond the range of the indicator on the alkaline side by dipping it into an M/10 solution of NH_4Cl, which on account of hydrolysis has a strongly acid reaction. That the alkalinity is due to ammonia rather than to some other substance secreted by the glands may be shown: (*a*) by the use of Nessler's reagent; and (*b*) by the fact that thorough aeration of the liquid greatly diminishes its alkalinity.

If the frog's skin is turned right side out instead of inside out, the results are much less striking. Clear evidence of selective penetration by NH_3 has been obtained with this arrangement a number of times, though frequently it could not be seen at all, and in no case was it so striking as with the skin turned in the reverse direction. The reason for this difference is not altogether clear at present. Perhaps the glands in the skin are in some way concerned, though the facts that the characteristic result may at times be obtained with the skin turned in either direction and that it may be obtained with eel's skin which is said not to contain glands, as well as with single cells of both plant and animal origin, make it fairly certain that it is not primarily a glandular phenomenon.

The difference in the behavior, in the presence of living cells, of three solutions of the same pH and the same salt concentration but containing respectively NaCl, NH_4Cl plus NH_4OH, and $NaHCO_3$ plus CO_2, may be shown very clearly by the "artificial cell" method, using phenol red as the indicator and starting with all of the solutions concerned (both internal and external) at a pH of 7.2. At the end of a few minutes, if the internal solution is gently stirred, the colors

in the three tubes will be found to be orange, deep red, and lemon-yellow, respectively. The author has used this experiment with success as a class exercise which never fails to give striking results.

As to the significance of the observations which have been described, it may be mentioned that NH_3, like CO_2, is a substance probably formed by all living cells. That a differential permeability effect should at times be responsible for differences in reaction between body fluids and cells and even between different parts of the same cell is by no means unlikely. That ammonium salts may, as a matter of fact, behave in this way is indicated by the work of Haldane[5] who finds them to be extremely effective in producing in the human body the changes generally described by the term acidosis. In the same connection, the views of Mathews[6] as to the possible rôle of ammonium salts in the formation of HCl in the mammalian stomach may be mentioned. Probably other physiological peculiarities of ammonium salts may ultimately be found to be connected with their behavior as here described.

To state briefly the general conclusions to be drawn from these experiments, it appears that NH_4OH among bases occupies much the same position that carbonic acid does among acids. In both cases the intracellular pH depends not on the pH of the surrounding medium but rather on the absolute concentration of the free base or free acid as the case may be. It follows, therefore, that while the determination of the pH values of body fluids, culture media, etc. is of the greatest importance, it is necessary to keep in mind the fact that these values do not always enable a prediction to be made as to the effect of the medium on living cells. In certain cases the results may be the exact opposite of those which might be expected, namely, in the cases where an alkaline solution containing CO_2 brings about intracellular acidity and where an acid solution containing NH_4OH brings about intracellular alkalinity.

[5] Haldane, J. B. S., *J. Physiol.*, 1921, lv, 265.

[6] Mathews, A. P., Physiological chemistry, New York, 1915, 374.

SUMMARY.

1. It may be shown by means of cells of the flowers of a hybrid *Rhododendron* which contain a natural indicator, by means of starfish eggs stained with neutral red, and by means of an "artificial cell" in which living frog's skin is employed that increased intracellular alkalinity may be brought about by solutions of a decidedly acid reaction which contain ammonium salts.

2. These results are analogous to those previously obtained with the CO_2-bicarbonate system, and depend on the facts: (*a*) that NH_4OH is sufficiently weak as a base to permit a certain degree of hydrolysis of its salts; and (*b*) that living cells are freely permeable to NH_4OH (or NH_3?) and not to mineral and many organic acids, and presumably not at least to the same extent to ammonium salts as such.

A MICRO INJECTION STUDY ON THE PERMEABILITY OF THE STARFISH EGG.

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It is well known that selective permeability, or semipermeability, is one of the essential characteristics of the living cell. So far, however, there is no evidence as to whether the semipermeability of protoplasm is a property of its entire mass or of its surface only.

Apparently the only means by which the action of substances on the interior alone of protoplasm may be studied is by injection. Animal cells can be injected by using the very fine glass pipettes and the mercury injection method which Barber devised for bacteriological work. I have used this method. The pipettes, both as regards their size and the ease of making, leave nothing to be desired. The method, however, is not only very difficult, but is unsatisfactory, owing to the fact that the pressure required for injection depends upon the expansion of mercury by heat, and this cannot be instantly controlled. Kite[1] tried it, but substituted for most purposes the far cruder method of blowing into his pipettes through a rubber tube. This operation necessitates larger pipettes than can be properly used for cell injection. The erroneous conclusions arrived at by Kite were due not only to the difficulty of the procedure, but mainly to the extraordinary ability of protoplasm to form films over torn surfaces. Pushing a pipette, especially a comparatively large one, into an egg cell frequently causes the surface of the cell to become invaginated and thus forms a deep pocket. The tip of the pipette, even if it should finally break through the surface, is apt to be separated from the protoplasm of the interior by the formation of a new surface film continuous with the original surface of the cell. Kite apparently did not guard against this contingency, and his experi-

[1] Kite, G. L., *Biol. Bull.*, 1913, xxv, 1.

mental results indicate that his solutions never actually entered the protoplasm of the cell. The injected fluid simply seems to have filled the bottom of a pocket and then flowed down the side of the pipette to the exterior. When Kite says that he found no difference in permeability, whether a substance be applied to the surface of a cell or to any spot in its interior, he was perfectly correct. The spot in the interior was an infolding from the surface of the cell. At best, he was only comparing the permeability of the original surface of the cell with that of a newly formed surface film which surrounded the tip of his pipette.

I have recently succeeded[2] in devising a simple but efficient piece of apparatus with which one can accurately and easily control the injection of fluids through a micro pipette having an aperture of less than 1 micron in diameter. The pipette, when properly made, tapers rapidly to a tip with a sharp cutting edge. The apparatus is so constructed that the pipette can be quickly changed. By keeping in mind the ease with which protoplasmic surface films are formed, one can, with this method, readily and accurately inject fluids directly into the interior of the protoplasm of a cell.

This summer Jacobs kindly set at my disposal a manuscript which the reader will find in this number of this Journal, in which are described the interesting results that were obtained by immersing neutral red vitally stained starfish eggs in ammonium chloride, and in sodium bicarbonate solutions. Jacobs found that a 1/2 M NH_4Cl solution, which is sufficiently acid to redden neutral red, will cause the neutral red within the eggs to turn yellow, indicating the entrance into the eggs of NH_3 and not of HCl. He also found that neutral red stained eggs will turn a deeper red when immersed in an alkaline solution of 1/2 M $NaHCO_3$ charged with CO_2, indicating the entrance into the eggs of CO_2 and not of NaOH.

Jacobs' results confirm those of Loeb,[3] Bethe,[4] Warburg,[5] Harvey,[6]

[2] Chambers, R., *Anat. Rec.*, 1922, xxiv, 1.

[3] Loeb, J., *Biochem. Z.*, 1909, xv, 254.

[4] Bethe, A., *Arch., ges. Physiol.*, 1909, cxxvii, 261.

[5] Warburg, O., *Z. Physiol. Chem.*, 1910, lxvi, 305.

[6] Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507; *Internat. Z. physik. Chem. u. Biol.*, 1914, i, 463.

and Crozier[7] that weak acids and bases freely penetrate living cells, whereas strong acids and bases do not. This is presumably because of their solubility in the organic solvents (lipoids) of the protoplasmic surface layer.

I have injected the solutions which Jacobs used into starfish eggs vitally stained with neutral red, and obtained decided and consistent results, which show that HCl and NaOH will permeate protoplasm freely as long as there is no protoplasmic film to serve as a barrier. The semipermeability of protoplasm, in all probability, depends upon the surface film having properties different from those of the continuous internal protoplasm.

EXPERIMENTS.

Mature starfish (*Asterias forbesii*) eggs were vitally stained with neutral red. They were then placed in a hanging drop in Barber's moist chamber, and those eggs selected which showed a neutral tint of an orange-red hue. All the experimental work was done under a Leitz $\frac{1}{7a}$ oil immersion objective and ocular 15. This objective gives a remarkably long working distance together with a sharp definition that allows of the use of high powered oculars.

Treatment with 1/2 M NH_4Cl.

Eggs were placed in a hanging drop of 1/2 M NH_4Cl in which, as Jacobs has shown, their vitality remains unimpaired for a period of 10 to 15 minutes. With a microdissection needle deep cuts were made in the eggs. The cut surfaces were immediately bounded by surface films continuous with the surface of the egg, and no injurious effect of the surrounding medium was noticeable. During this time, the neutral red within the egg, gradually turned yellow.

This experiment indicates that the NH_4Cl does not prevent the formation of films over the cut surfaces of the egg, and also that the solution will not, within the time limits of the experiment, penetrate those films.

The interior of stained eggs was made to flow out in a drop of 1/2 M NH_4Cl by the following means. The egg was torn at one spot on its surface and then caught on the other side and pulled to the edge of the drop. In every case the rapidly outflowing interior turned rose-red upon coming into contact with the surrounding solution and cytolyzed into a frothy semisolid mass. The change from an orange color to a red, with an accompanying cytolysis, extended from the out-

[7] Crozier, W. J., *J. Biol. Chem.*, 1916, xxiv, 255.

flowing area into the egg itself, and spread to the original cortex until the entire egg was cytolyzed.

This experiment shows that if the egg be torn in such a way as to cause its interior to flow out rapidly, no surface film forms. The NH_4Cl at once penetrates the protoplasm which undergoes the characteristic color change and cytolysis.

Stained eggs were placed in a hanging drop of sea water and 1/2 M NH_4Cl injected under the egg membrane. This is fairly easy to do in the unfertilized egg but more so in the fertilized egg in which the membrane has already lifted off as the so called fertilization membrane. In the unfertilized egg, the injected solution at first bulges the membrane giving rise to a localized blister, and then usually spreads quickly over the egg, lifting the membrane from its entire surface. The permeability of the egg to the NH_4Cl is not affected by this treatment. This demonstrates that it is not the egg membrane which protects the egg from the NH_4Cl, but the actual surface film of the protoplasm lying under the membrane.

Stained eggs in a hanging drop of sea water were punctured with a glass pipette having an aperture of 1 micron in diameter, and a minute quantity of 1/2 M NH_4Cl injected directly into the interior of the egg. The injected area immediately changed from an orange to a rose-red color, and then underwent cytolysis. The color change and accompanying cytolysis spread from the injected area. In some cases this spread was arrested by the formation of a surface film which converted the injected and disintegrated area into a vacuole. In other cases the cytolysis spread till it reached the cortex which disintegrated from within outward.

This experiment demonstrates that 1/2 M NH_4Cl, which causes an alkaline color change within eggs immersed in it, will, when injected into the interior of the eggs, produce the acid color change and accompanying cytolysis which characterizes the presence of HCl.

Treatment with 1/2 M $NaHCO_3$ + CO_2.

Stained eggs were cut and torn in a hanging drop of the $NaHCO_3$ solution. In contrast to the reaction in the presence of NH_4Cl there was no tendency for the formation of surface films over their cut surfaces. The protoplasm simply flowed out and was dispersed in the solution, the color changing meanwhile from red to yellow.

Injection of $NaHCO_3$ beneath the egg membrane of eggs in sea water had no other effect than that produced upon eggs by immersing them in the solution; *viz.*, deepening of the red color in the egg owing to the selective penetration of CO_2.

Stained eggs were injected with the $NaHCO_3$ solution. The injected area immediately turned yellow, and cytolysis with liquefaction took place. No surface film formed about the cytolyzing area, and the yellow color spread throughout.

CONCLUSION.

The experiments with the NH_4Cl are similar to, and corroborate micro injection experiments performed in connection with some work on mustard gas in which the writer[8] collaborated. Eggs immersed in sea water containing decomposed mustard gas, at a certain low concentration are not affected. If, however, the solution be injected, the egg quickly cytolyzes owing to the free HCl present.

A similar impermeability of the protoplasmic surface film to certain substances was also encountered in injection work on *Amœba*.[9] *Amœbæ* immersed in an aqueous solution of eosin will not take the stain till after death. On the other hand, the eosin, when injected into the *Amœba*, quickly permeates the protoplasm, to be arrested only at the surface.

The semipermeability of a living cell appears primarily to be a function of its surface film. It is immaterial whether this film be that of the original cortex of the cell, a film newly formed over a cut surface, or a film that surrounds an artificially induced vacuole within the cell. As long as such a surface film exists neither the acid group of the NH_4Cl nor the alkaline group of the $NaHCO_3$ can, within certain concentration limits, penetrate the protoplasm. These solutions, if injected beneath the surface film, however, will produce their characteristic effects upon the protoplasm.

[8] Lillie, R. S., Clowes, G. H. A., and Chambers, R., *J. Pharmacol. and Exp. Therap.*, 1919–20, xiv, 75.

[9] Chambers, R., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 66.

THE RELATION OF THE REACTION AND OF SALT CONTENT OF THE MEDIUM ON NITRIFYING BACTERIA.*

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The literature of soil bacteriology is replete with assertions to the effect that soil bacteria of value for the maintenance of an appropriate soil condition need an alkaline medium for their proper development. This has been repeatedly emphasized, particularly in connection with the nitrifying and the nitrogen-fixing flora of the soil. Actual evidence substantiating such assertions has never been adduced from experiments, or at least, it has never been presented. This situation is, in part, explained by the vagueness of our old conceptions with regard to the real nature of alkalinity and acidity as applied to biological media. It is natural, therefore, that studies such as those needed to give the subject a proper status should have lagged behind the rapid development of the methods for determining hydrogen and hydroxyl ion concentrations in the last 8 to 10 years. Even such a recent investigation as that of Stephenson (1) on the relations of nitrification to acidity in soils takes no account of the

* Since this paper was written, there has come to our notice the study of Meyerhof (Meyerhof, O., *Arch. ges. Physiol.*, 1916, clxiv, 353), on the respiration of nitrate-forming bacteria. Meyerhof found that the optimum pH for the nitrate former was between 8.3 and 9.3. This is considerably below the figure obtained by us as described in this paper. Moreover, Meyerhof's establishment of definite lethal points at pH values of 5.6 and 10.3 for hydrogen and hydroxyl ion, respectively, seem to us to be based on insufficient evidence, inasmuch as we show in this paper that the strain of the nitrifying bacteria as determined by its source is an important factor of the lethal point of reaction. Finally, the actual resistance to hydroxyl ion as judged by ability to live (even though inactive), is found by us to be much greater than that indicated by Meyerhof's results. In general, however, it is gratifying to find that the two studies confirm each other on the point that nitrifying bacteria are highly resistant to hydroxyl ion and are, in general, less resistant to hydrogen ion.

actual hydrogen ion concentrations involved, but only of acidity expressed in the terms of more than a decade ago.

In view of the foregoing, we deemed it necessary to determine, if possible, just what the relations of nitrifying bacteria to the reaction of their medium may be. In addition, another subject connected with the nitrifying bacteria seemed to call for our attention, and that was the relations of nitrifying bacteria to high salt concentrations. One of us (2) had already investigated the latter subject by using soils as media. That work, however, was done some years ago without due appreciation of the uncontrolled variables involved in the use of soils as media. For that reason, it seemed highly desirable to study the subject of salt concentrations again and this time in culture solutions of simple and known concentration.

Experiments on Reaction of the Medium.

The culture solution employed in these experiments was constituted as follows:

1,000	cc. distilled water.
1	gm. K_2HPO_4.
1	" NaCl.
0.5	" $MgSO_4$.
1	drop of 10 per cent solution of $FeCl_3$.

To this solution was added, as needed, 1 gm. of $(NH_4)_2SO_4$ per liter for nitrite formation cultures and 1 gm. of $NaNO_2$ per liter for nitrate formation cultures. Varying amounts of NaOH and H_3PO_4 were added to the different series of cultures to provide a wide range of hydroxyl and hydrogen ion concentrations, respectively. The actual concentrations of these ions in every culture were determined either by the hydrogen electrode or by the colorimetric method, of Clark and Lubs, and in many instances by both methods, used so as to check each other. Determinations of the hydrogen and hydroxyl ion concentrations were made at frequent intervals during the incubation period. When solutions of low concentration of hydrogen ion were desired, K_2HPO_4 was employed. In order to insure a good buffer effect, the 14.25 normal NaOH which we used, was made up with a K_2HPO_4 solution, instead of with distilled water.

Difficulties encountered in attempts to obtain pure cultures of the nitrifying bacteria led us to adopt the use of a crude, but invigorated culture in each case, which was produced by numerous successive transfers into fresh media from a strong soil culture in the solutions above described. The media were distributed in 25 cc. portions in 250 cc. Erlenmeyer flasks and plugged and sterilized as is customary. The cultures were run in quadruplicate and the series in duplicate.

Incubation was carried out at a temperature of 28°C. Only qualitative tests were made for nitrites and nitrates, the Trommsdorff reagent and the diphenylamine tests being employed. At the end of the first 5 days of the incubation period and on every succeeding day, every culture was tested. Sterile distilled water was added from time to time to maintain constant concentrations. In the three different series of our experiments, different soils were employed for obtaining the necessary bacterial cultures. These soils were a garden soil, a blow-sand soil, and a peat soil. We assumed that the enrichment cultures which we obtained by several months of culturing by the Gibbs' method (3) could be justifiably used because any contaminating organisms present could not be expected to exert any influence on the nitrifying bacteria in the peculiar solutions used. Since the results obtained were similar in the different soils, we present here only the data for the garden soil organisms. In the case of the peat soil, we give the data on nitrite formation separately, since they differed from those of the garden soil. In Table I will be found the results obtained with the nitrate-forming bacteria operating in mixed cultures, but in solutions which presumably prevent other bacteria from working. In Table II, we present the results obtained from the garden and the peat soil cultures of the nitrite-forming organisms. Where bacterial action was positive, indication thereof is given in the tables by a plus sign; where it was negative by a minus sign.

The data in the tables furnish most interesting and unexpected results. In comparing the hydrogen and hydroxyl ion concentrations, expressed in our tables by pH values, for the freshly inoculated cultures with those first showing nitrification, we note the very striking fact that both nitrite- and nitrate-forming organisms from the garden soil can withstand extremely high concentrations of hydroxyl ion,

in one case pH 13.0 and in the other 13.1. This exceeds the resistance of any living organism, of which we have knowledge, to the effects of alkalinity. In the second place, the comparison in question confirms the findings of several investigators to the effect that living cells modify very rapidly the reaction of the media into which they are introduced. This seems to be a universal phenomenon among

TABLE I.

Series 1. Nitrobacter Garden Soil.

pH values.		Nitrification.
At beginning.	At time of nitrification.	
14	14	−
13.8	13.8	−
13.4	13.4	−
13.1	10.0	+
12.8	9.9	+
12.7	10.3	+
12.5	9.6	+
12.3	9.8	+
11.6	9.6	+
11.1	9.4	+
10.5	9.0	+
9.5	8.6	+
8.8	8.6	+
8.5	8.4	+
7.2	6.6	+
7.1	6.6	+
7.0	6.6	+
6.6	6.5	+
6.2	6.2	+
5.6	5.6	+
5.4	5.3	+
5.3	5.3	−

living cells. While, therefore, both the nitrite- and nitrate-forming bacteria originating from the garden soil could live and function in solutions whose pH was 13.0 and 13.1, respectively, at the time when they began to yield their characteristic products they had reduced the pH value of their media to 10.3 in one case, and to 10.0 in the other. Even that corresponds to a hydroxyl ion concentration, however, which is in excess of that tolerated by any other organisms

of which we have knowledge. It is significant, further, that at the lower concentrations of NaOH employed, there was relatively little

TABLE II.

pH values.		Nitrification.
At beginning.	At time of nitrification.	
Series 2. *Nitrosomonas* garden soil.		
13.6	13.6	−
13.4	13.4	−
13.0	10.0	+
10.0	9.3	+
9.6	9.0	+
9.5	8.8	+
9.0	8.6	+
8.0	7.8	+
7.8	7.6	+
7.3	7.2	+
6.8	6.6	+
6.4	6.4	+
6.2	6.2	+
6.0	6.0	+
5.4	5.4	+
4.7	4.7	−
4.3	4.3	−
4.2	4.2	−
Series 3. *Nitrosomonas* peat soil.		
13.0		−
10.0		−
9.5	9.3	+
8.0	7.8	+
7.2	7.1	+
6.8	6.8	+
6.4	6.3	+
6.2	6.2	+
6.0	6.0	+
5.4	5.2	+
4.7	4.6	+
4.3	4.2	+
4.2	4.1	+

change in the hydroxyl ion concentration from the initial value to that characterizing the solution when nitrite formation was observed.

That was not so true of nitrate formation. The extreme resistance of these organisms as noted above was further tested after they had shown their characteristic activities in the cultures of pH values of 13.0, and had reduced these cultures to pH 10.0; more alkali was then added, bringing the hydroxyl ion concentration up to the original value, but nitrification was thereby stopped. That the process did not later repeat itself as before was doubtless due to the high osmotic concentration of the solution which resulted from the addition of these large quantities of alkali. In the case of the peat soil organisms, we find much less resistance to hydroxyl ion concentration than in the garden soil and no nitrate formation is found at pH values above 9.5. Even those organisms, however, produced nitrites from ammonia at a pH of 9.3, and while this shows considerable variability as compared with the nitrifying bacteria from the garden soil, it strengthens the evidence on the most striking point presented in our data, to the effect that the resistance of nitrifying bacteria to high hydroxyl ion concentrations is exceptional in the living world.

On the acid side of the neutral point, our observations furnish the following data. The organisms from the garden soil stop the production of both nitrites and nitrates at pH values below 5.4. Again we find a difference between the organisms from the garden and the blow-sand soil, which always behaved exactly alike, and those from the peat soil. Not only did the latter go on producing nitrites from ammonium salts in solutions with a pH below 5.4, but continued doing so at a pH of 4.1 which was the most acid medium employed by us. Doubtless their resistance to acidity can be shown to be much greater, but new experiments on that point would have to be arranged, providing for the use of an acid which dissociates to a much greater degree than the H_3PO_4 which we used, so that small increases in concentration of acid would suffice to allow the requisite range of hydrogen ion concentration. Since the reason for our using H_3PO_4 must be obvious to the reader, no further discussion of those points is necessary here. It may not be out of place, however, to mention a difficulty in a problem like ours in connection with the use of high concentrations of acid; that is, that nitrous acid is oxidized under those conditions to nitric acid without the intervention of the bacteria. That necessarily limits us to certain ranges of hydrogen ion concentration.

Recurring now to the results obtained with the organisms of the peat soil, it becomes evident that they cannot withstand as much hydroxyl ion but can resist much more hydrogen ion than the nitrifying bacteria from the other soils. Since the peat soil is naturally acid in reaction (pH about 4.6), it is of interest to ascertain whether in this case a marked impression is made upon an organism by its environment even though such impression does not produce a change in the character of its fundamental functions. The nitrifying bacteria will probably be traced back some day to a common source. If that should be the case, our data will furnish one step in the line of reasoning which may prove the marked influence of the environment on living cells.

As regards a further comparison between the nitrite and nitrate formers, it should be added that, while our data are not conclusive on that point, they would seem to indicate, in general, that the nitrate-forming bacteria are slightly more resistant to alkalinity than the nitrite-forming bacteria.

It seems of interest to consider the rate at which the nitrate-forming bacteria produce nitrates from nitrites under varying concentrations of hydrogen and hydroxyl ion, other conditions being equal throughout. Fig. 1 gives in graphic form the results of such a study. The curve indicates the amount of $NaNO_2$ (in milligrams) which was oxidized to nitrate at different pH values of the culture solutions. It will be seen from the curve that the qualitative results of the tables are confirmed, the maximum velocity of reaction being noted at pH 10.0. This, therefore, seems to be the optimum point for the speedy oxidation of nitrite to nitrate. The rate of increase from pH 6.1 is extremely rapid up to pH 7.1. It then becomes much slower until the maximum increase is reached at pH 10. Beyond the latter point, there is a gradual drop in the rate until an initial pH of 13.1 is reached, in which culture 237 mg. of $NaNO_2$ are oxidized in 32 days.

The Resistance of Nitrifying Bacteria to High Salt Concentrations.

This part of the investigation was preliminary in nature and sought to establish some definite ranges of salt concentration within

which nitrification by the organisms in question proceeds actively and without which it ceases completely. Therefore, three sodium salts were employed at a few concentrations only with the results indicated in Table III. The plus sign indicates active nitrification;

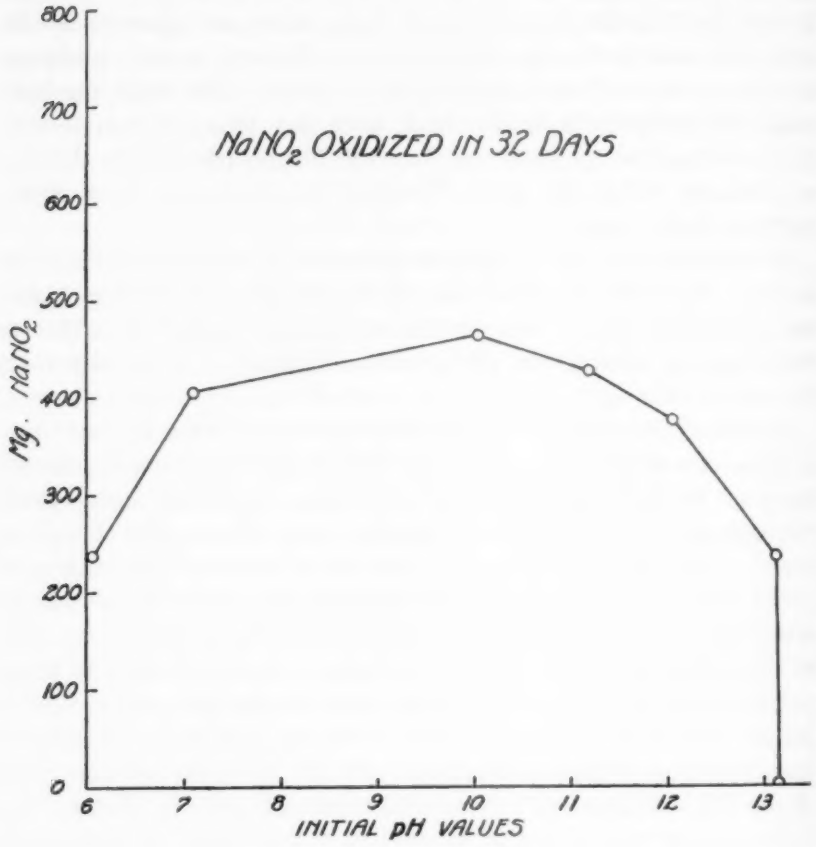

FIG. 1.

the minus sign, no nitrification. The culture solutions were made up in Erlenmeyer flasks as in the experiments described above and the organisms were grown (not in pure, but in invigorated crude cultures) in thin layers of solution. The salts were added as above indicated.

The data of Table III seem to justify the conclusion that the toxicities of NaCl and Na_2CO_3 to nitrifying bacteria are of about the same order of magnitude, but that Na_2SO_4 is not nearly so toxic as either of the other salts. The problem is more complicated in the case of the Na_2CO_3 series inasmuch as the hydroxyl ion concentration was greater in the solutions containing it than in the solutions supplied with the other salts. This effect must, however, have been offset more or less completely by the marked buffer action of the nitrifying cells which is discussed in the first part of this paper. The results

TABLE III.

	Nitrification.
NaCl series.	
10,000 parts per million (0.171 N)	+
20,000 " " " (0.282 N)	−
Na_2CO_3 series.	
5,000 parts per million (0.094 N)	+
10,000 " " " (0.188 N)	+
20,000 " " " (0.276 N)	−
Na_2SO_4 series.	
10,000 parts per million (0.140 N)	+
20,000 " " " (0.280 N)	+
25,000 " " " (0.350 N)	+
30,000 " " " (0.420 N)	+
35,000 " " " (0.990 N)	−

in Table III suggest the immediate need for more detailed experiments along this line, which we hope to carry through. It should be added, however, that since work of the kind reported in this paper is the first reported for simple culture solutions with nitrifying bacteria, and since such cultures are much simpler and involve fewer variables than soil cultures, our results must be regarded as more reliable than the earlier ones obtained by one of us and by other investigators who have dealt with the problem more recently. While the findings of the present paper agree with earlier ones regarding the relative toxicity of NaCl and Na_2SO_4 to nitrifying bacteria, the

other features of the results are very different and for the reason given above should be considered more dependable. In all cases, it is very striking to note the marked tolerance for high salt concentrations manifested by the nitrifying bacteria.

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1. Stephenson, R. E., Nitrification in acid soils, *Iowa Agric. Exp. Station, Research Bull., 58*, 1920, 329.
2. Lipman, C. B., Toxic effects of "alkali salts" in soils on soil bacteria. II. Nitrification, *Centr. Bakt., 2te Abt.*, 1912, xxxiii, 305.
3. Gibbs, W. M., The isolation and study of nitrifying bacteria, *Soil Sc.*, 1919, viii, 427.

THE EQUIVALENCE OF AGE IN ANIMALS.

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Donaldson[1] in his comparison of the white rat with man in respect to the growth of the entire body, assumed that the rat at the age of 3 years, is comparable in age to a man of 90 years, and that the same proportional relations hold for fractions of this period. The duration of life was employed as the basis of comparison. The objection against employing the duration of life as a basis for estimating the equivalence of age consists, as Donaldson pointed out, in the incomplete information at our disposal concerning the onset of old age and duration of life. Now it is well known that growth is periodic or cyclic. The warm blooded animals studied go through three periods or cycles of growth which may be termed infantile, juvenile, and adolescent cycles; or first, second, and third cycles. The curve of each cycle is symmetrical around its center, the maximum of the cycle. According to theories of growth developed by Loeb,[2] Robertson,[3] and Ostwald,[4] growth under normal conditions is limited by a series of consecutive chemical reactions, and the middle, or maximum, of each cycle represents the middle of the respective limiting chemical reaction. Assuming that these theories describe the facts, then the maxima of the corresponding cycles in different animals represent equiva-

[1] Donaldson, H. H., A comparison of the white rat with man in respect to the growth of the entire body, Boas anniversary volume, New York, 1906, 5.

[2] Loeb, J., The dynamics of living matter, New York, 1906, 58; *Biochem. Z.*, 1906, i, 34; *Biol. Centr.*, 1910, xxx, 347.

[3] Robertson, T. B., *Arch. Entwicklungsmechn. Organ.*, 1907–08, xxv, 581; Principles of biochemistry, for students of medicine, agriculture and related sciences, Philadelphia, 1920.

[4] Ostwald, W., Vorträge und Aufsatze über Entwicklungsmechanik der Organismen, Leipsic, 1908, v, cited by Loeb (1910).[2]

lent stages of growth; that is, equivalent physiological ages as far as growth is concerned. The maxima of the cycles may, therefore, be taken as points of reference in the course of life of animals, and the conceptional ages of these maxima may be employed for estimating the equivalence of age in different animals.

The age of maxima of the cycles may be conveniently determined by plotting the gains in weight per unit time at different ages against the corresponding ages. Such plots are shown in Figs. 1 and 2 which are self-explanatory.[5] From these plots it is seen at once that the maximum of the third cycle in the rat is 64 days of age, in the mouse 42 days of age, in man about 14 years of age; in other words, a rat of 64 days of age is physiologically as old as a mouse 42 days of age, a man 14 years of age, and so on for the other animals as shown in Table I. The maxima of the first and second cycles, and the junctions between the cycles, similarly represent equivalent physiological ages.

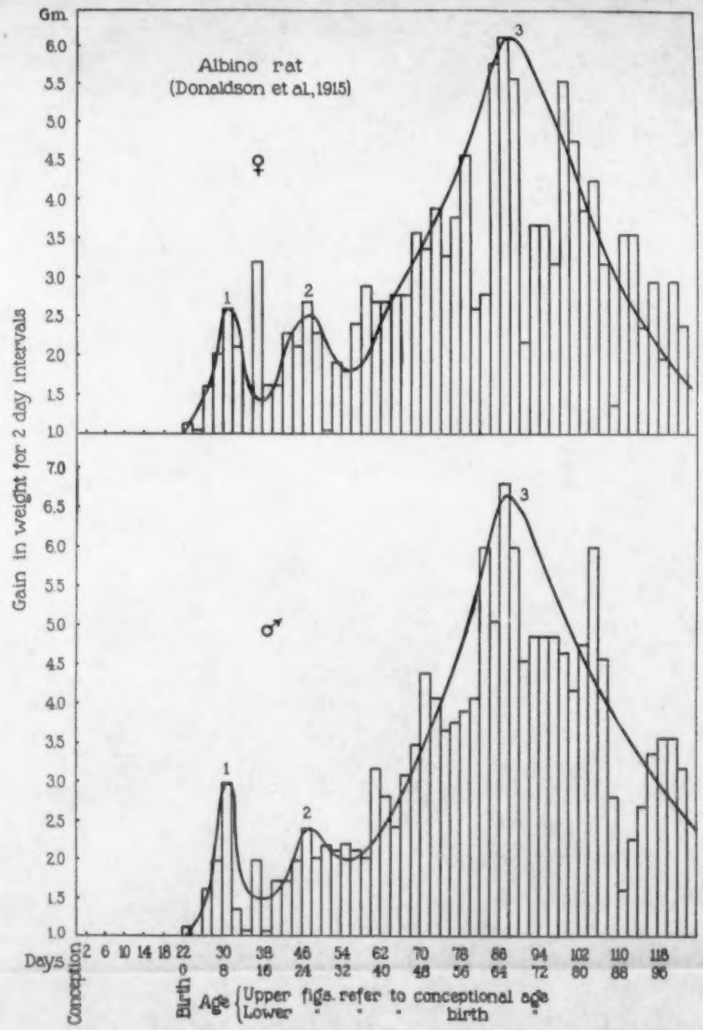
If there is the same linear relation between the duration of the period of growth and the duration of life in different animals then in order to estimate the equivalent physiological ages, for example the duration of life in different animals, it is only necessary to multiply the conceptional ages of the third maxima by some constant. Thus multiplying the conceptional ages of the third maxima of the rat and mouse by 13 gives 3.0 and 2.15 years respectively which are practically the values obtained by Donaldson[1] and Robertson and Ray[6] for the duration of life of these animals. Taking 13 as the constant of the linear equation connecting the duration of life with the conceptional age of the maxima of the third cycles, we obtain the duration of life of different animals as shown in Table II.

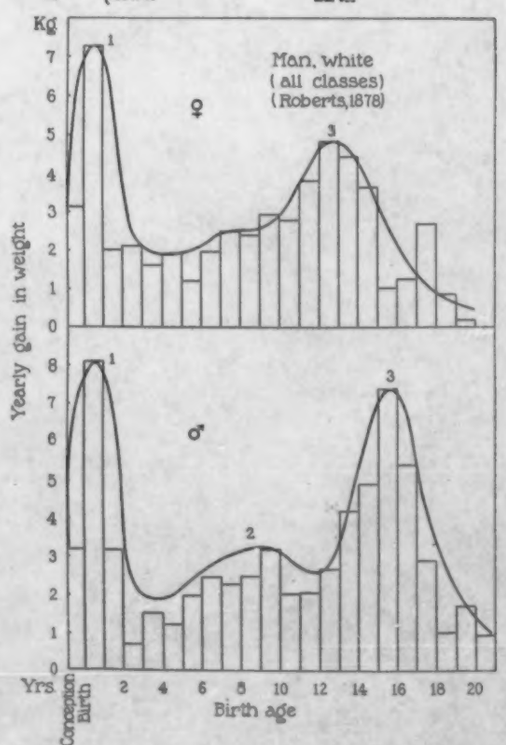
Table II shows that, with the exception of man and fowl, the calculated values for the duration of life of different animals are very close to the average actual duration of life under the most favorable conditions. The value for man, about 185 years, is probably too high, although a perusal of Metchnikoff's[7] discussion on longevity

[5] The sources of data on which the figures are based are given at the end of the article. Figs. 3 and 4 are omitted because of difficulty of reproduction.

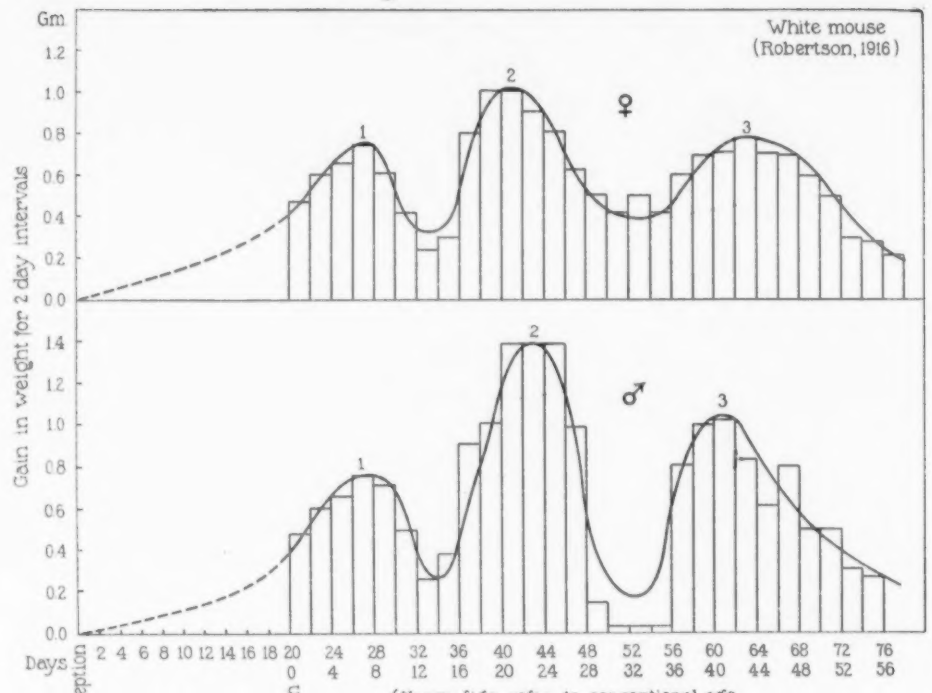
[6] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1920, xlii, 71.

[7] Metchnikoff, E., The nature of man, New York and London, 1903, 259.


Gm.
Albino rat
(Donaldson et al, 1915)
♀
♂
Gain in weight for 2 day intervals
Days
Conception
Birth
Age {Upper figs. refer to conceptional age
Lower " " " birth


Kg
Man, white
(all classes)
(Roberts, 1878)
♀
♂
Yearly gain in weight
Yrs
Conception
Birth
Birth age

Gm


Gm
White mouse
(Robertson, 1916)
♀
♂
1
2
3
Gain in weight for 2 day intervals
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Days
2 4 6 8 10 12 14 16 18 20 24 28 32 36 40 44 48 52 56 60 64 68 72 76
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56
Yrs
Conception
Birth
2 4 6 8 10 12 14 16 18 20
Birth age

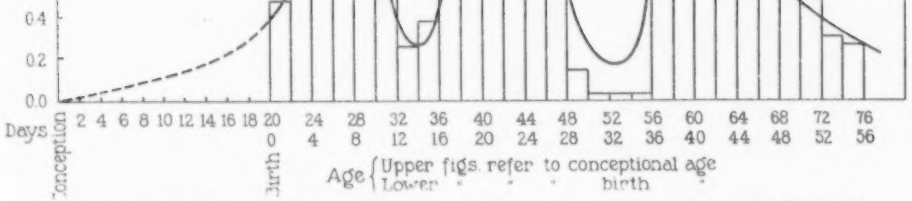
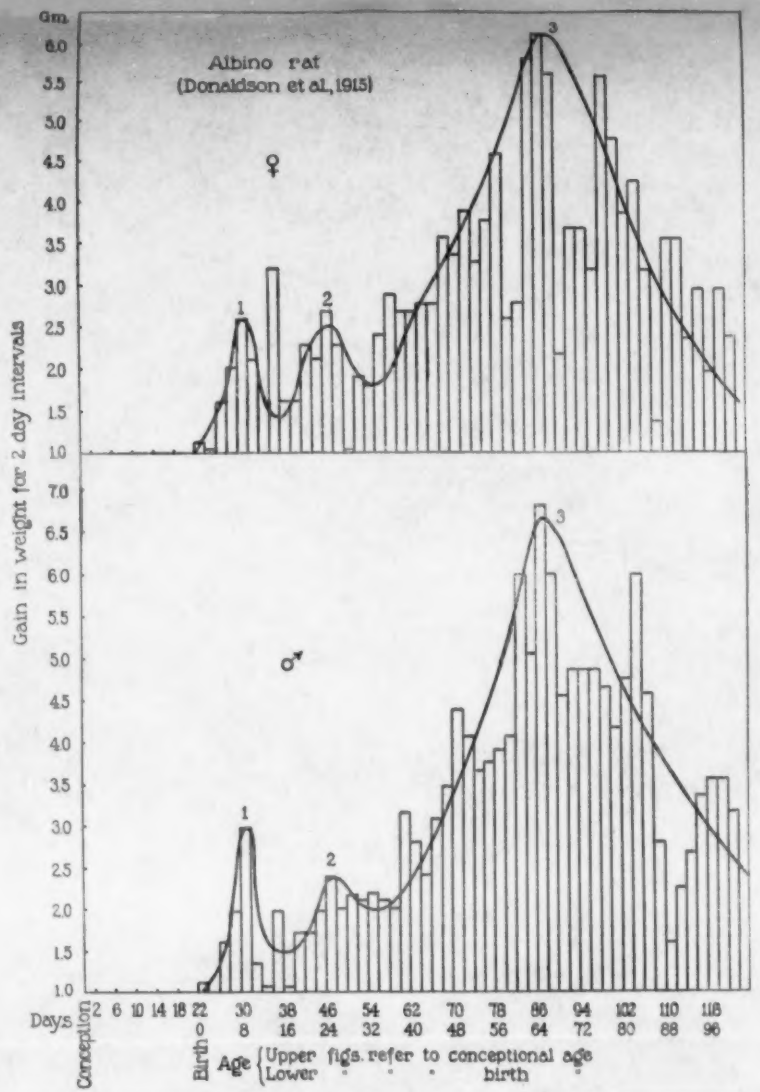
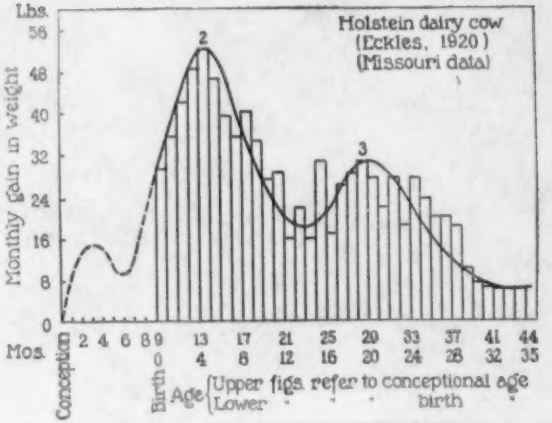
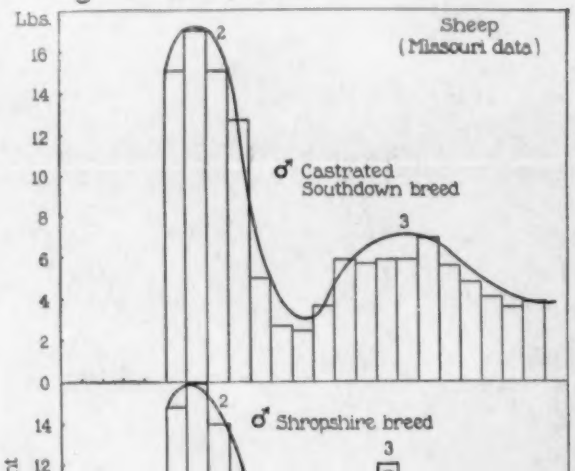
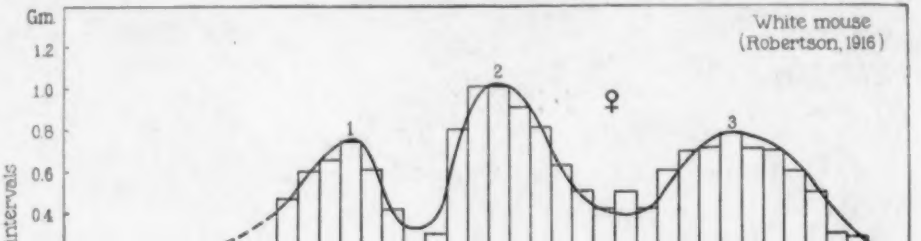


FIG. 1. Velocity curves of growth. Ordinates represent the velocities of growth; the height of rectangles represents gains in weight for the ages shown on the abscissæ. The maxima of the first and third cycles are presented on a base line of the same length.


Albino rat
(Donaldson et al, 1915)
♀
♂
Gm.
Gain in weight for 2 day intervals
Days
Conception
Birth
Age {Upper figs. refer to conceptional age
Lower " " " birth


Holstein dairy cow
(Eckles, 1920)
(Missouri data)
Lbs.
Monthly gain in weight
Mos.
Conception
Birth
Age {Upper figs. refer to conceptional age
Lower " " " birth


Sheep
(Missouri data)
Lbs.
♂ Castrated
Southdown breed
♂ Shropshire breed


White mouse
(Robertson, 1916)
♀
Gm.
intervals

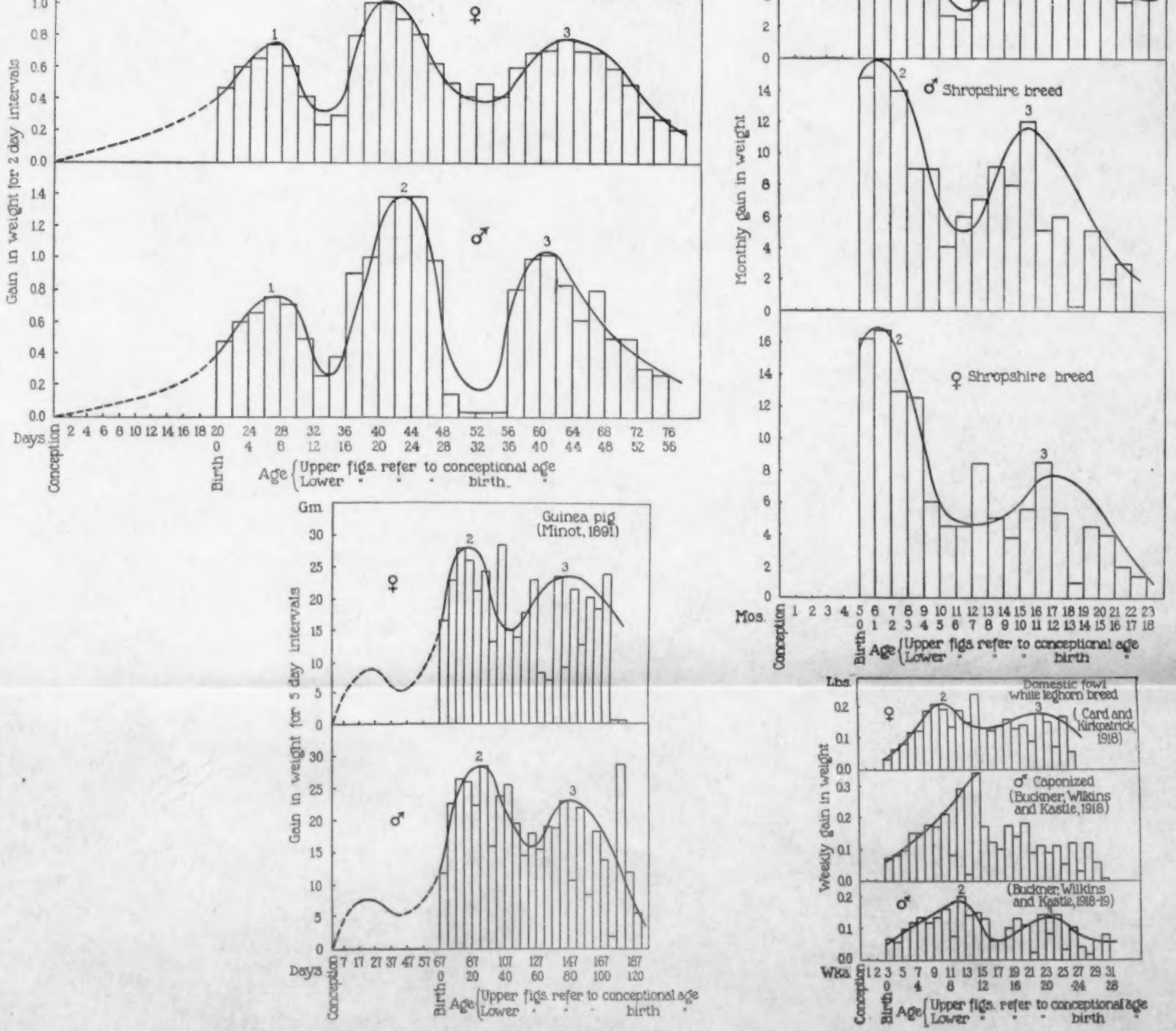


FIG. 2. Ordinates and abscissæ have the same meaning as in Fig. 1. The maxima of the second and third cycles are presented on a base line of the same length.

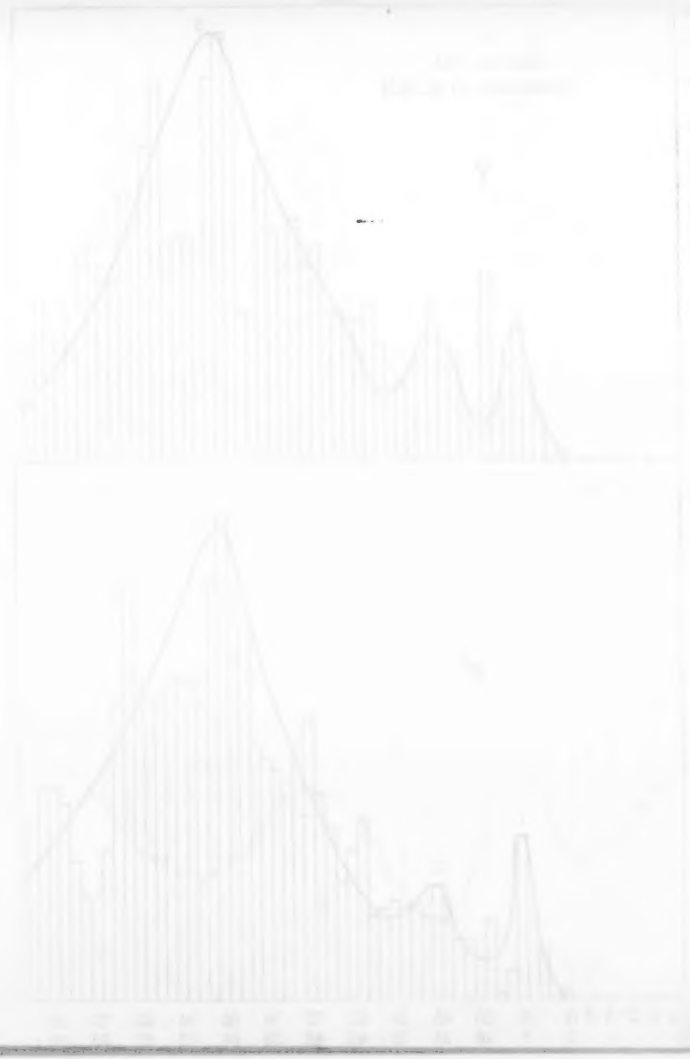


TABLE I.

Equivalence of Age.

Age of animals at the maxima of the three cycles and the junctions between the cycles.

Animal.	Age of maximum of first cycle.		Age of junction between first and second cycles.		Age of maximum of second cycle.		Age of junction between first and second cycles.		Age of maximum of third cycle.		Conceptional age at birth.
	Conceptional age.	Birth age.	Conceptional age.	Birth age.	Conceptional age.	Birth age.	Conceptional age.	Birth age.	Conceptional age.	Birth age.	
	days		*days*		*days*	*wks.*	*days*	*wks.*	*days*	*wks.*	*days*
Fowl.....	16	5 days before hatching.	18	3 days before hatching.	84	9	100	11	156	20	21
Guinea pig.....	23 (estimated)	44 days before birth.	32 (estimated)	35 days before birth.	85	2.5	115	7	145	11	67
		wk.		*wks.*							
Mouse.....	27	1.0	33	2	42	3	53	5	62	6	20
						mos.		*mos.*		*mos.*	
Ox (dairy cow)...	160 (estimated)	4 mos. before birth.	190	3 mos. before birth.	420	4.5	690	13.5	850	19	285
								wks.			
Pig.....							158	5			120
		days		*wks.*		*wks.*					
Rabbit.....	40	10	65	5	110	11	160	18	185	5	30
				days						*wks.*	
Rat.....	31	9	38	16	47	3.5	56	5	86	9	22
								mos.		*mos.*	
Sheep.....					195	6.5	360	7	480	11	150
		mos.								*yrs.*	
Man.....	345	2							5,300	13.7	285

does not make this value appear to be outside of the limits of possibility. On the whole, this product of the conceptional age of the maximum of the third cycle by 13, is a useful approximation as regards the maximum duration of life of these animals. Equivalence of age at any other period after the third cycle, may be similarly estimated by multiplying the conceptional age of the maximum of the third or adolescent cycle by an appropriate constant. Thus multiplying this age by 2, gives a value corresponding to the age of 28 years in man; multiplying by 4, gives a value corresponding to 55 years of age in man as shown in Table II.

TABLE II.

Equivalence of Age after the Age of the Third Cycle.

Values obtained by multiplying the conceptional age of the maximum of the third cycle by a constant indicated at the head of each column.

Animal.	×2	×3	×4	×5	×6	×7	×8	×9	×10	×11	×12	×13
Domestic fowl, *yrs.*	0.8	1.2	1.7	2.1	2.5	2.9	3.4	3.8	4.2	4.6	5.1	5.5
Guinea pig, *yrs.*...	0.7	1.0	1.4	1.8	2.2	2.6	3.0	3.4	3.8	4.2	4.6	5.0
Mouse, *mos.*.....	3.8	5.5	7.6	9.7	11.7	13.8	15.8	17.9	20.0	22.3	24.1	26.2
Ox (dairy cow), *yrs.*	3.9	6.2	8.3	10.9	13.2	15.5	17.8	20.2	22.5	24.8	27.2	29.5
Rabbit, *yrs*.....	0.9	1.4	1.9	2.5	3.0	3.5	4.0	4.4	5.0	5.5	6.0	6.5
Rat, *mos*.....	5.0	7.9	10.7	13.6	16.8	19.3	22.2	25.1	27.9	30.8	33.6	36.5
Sheep, *yrs*.....	2.2	3.5	4.8	6.2	7.4	8.8	10.1	11.4	12.7	14.1	15.4	16.7
Man, *yrs*.....	28	42	57	72	86	100	115	130	147	159	173	187

In addition to equivalence of age, the plots presented bring out a number of other facts. The corresponding cycles in different animals differ with respect to their relative duration and shape. Figs. 1 and 2 bring out the fact that the mouse and rat show a long preinfantile period not found in the other animals. Fig. 1 brings out the difference in shape of the second or juvenile cycle in man as compared to the other forms. The width of the juvenile cycle in man is, relative to the whole actively growing period, not very much greater than in the other forms. The striking difference between man and the other forms consists in the relative flatness of the curve of the juvenile cycle; its shape is not that of the curve of an autocatalytic reaction. The absolute gain in weight during the maximum of the juvenile cycle is greater than during any other period of life in all forms, except the

man and rat. In man the gain in weight is greatest during the maximum of the infantile period, or more specifically during the 2nd and 3rd months of life as shown in Fig. 5. In the rat, the gain in weight is greatest during the maximum of the third cycle. The third cycle in the rat is relatively also the widest, occupying about 60 per cent of the growth curve. The interpretation of these differences with respect to the relative shape and durations of the cycles is beyond the limits of our present state of knowledge. They only

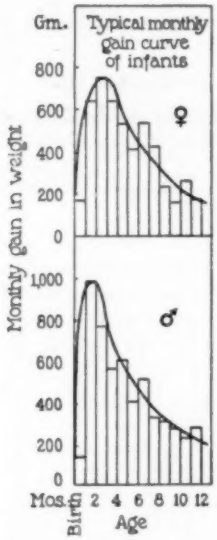


FIG. 5. Typical monthly gain curve of infants showing that the gain in weight is highest during the 2nd and 3rd months after birth.

show that either the limiting reaction of different cycles in different animals are fundamentally different, or that the conditions under which the reactions take place are different, or that both are different. The evolutionary significance of these differences will no doubt be illuminated by the accumulation of data on growth of related types of animals.

Another interesting difference brought out by the figures relates to the relative age at birth of different animals. Man, mouse, rat, and rabbit are born before the completion of the first or infantile cycle.

The other forms spend their infantile period *in utero*. The ox, sheep, pig, and guinea pig at birth are with respect to physiological age equivalent to man at the age of about 1.5 to 2 years; or to mouse and rat at 2 to 3 weeks. Man at birth is with respect to physiological age, equivalent to the ox at about 4 months before birth, that is about 5 months after conception; or equivalent to the guinea pig about 6 weeks before birth. Fig. 6 brings out the fact that the ox and guinea pig pass through a cycle *in utero*.

Fig. 2 also brings out the known fact that the maximum of the adolescent cycle usually comes earlier in girls than in boys. Fig. 1

TABLE III.

Age of Maximum of the Adolescent Cycle in Man.

Nationality.	Boys.	Girls.
	yrs.	*yrs.*
Chinese	15	13
Japanese	15	14
Philippine	15	13
Belgian	16	14
Danish	16	14
English	16	13
French	14.5	14
German	15	14
Italian	16	14
Swedish	16	14.5

is supplemented by Fig. 7 and Table III to bring out this fact, and incidentally to bring out the effect of race and economic conditions on the growth of children. The lower forms do not show appreciable difference due to sex. It is also interesting to note that the maximum of the adolescent cycle in girls comes at the same age as sexual maturity. According to Baldwin[8] the mode for sexual maturity in girls as shown by the first menstrual flow is 14 years. From Table III this is also the average age of the adolescent maximum.

[8] Baldwin, B. T., The physical growth of children from birth to maturity, *Univ. Iowa, Studies in Child Welfare*, 1921, i, 188.

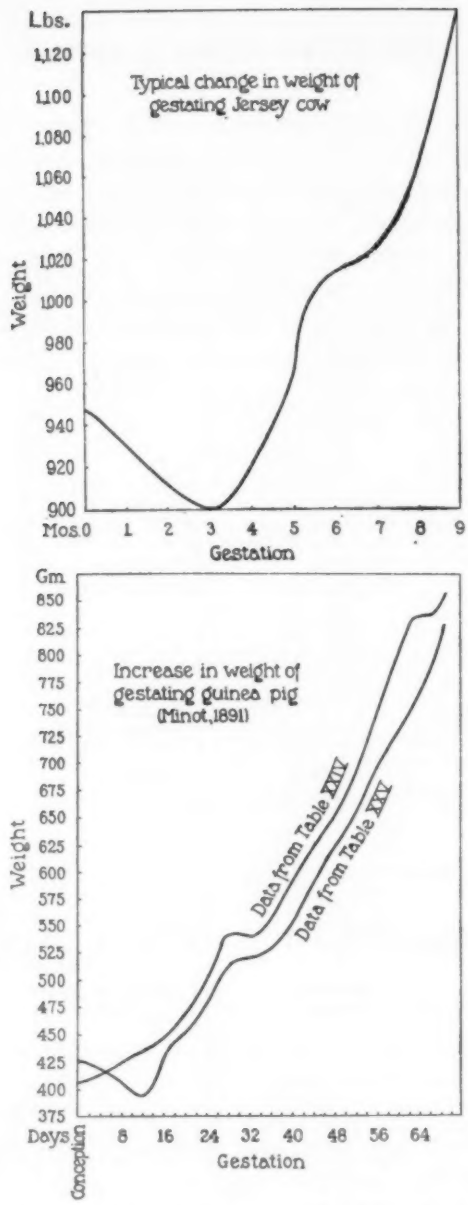


FIG. 6. Change in weight of gestating cow and guinea pig to illustrate the fact that the ox and guinea pig pass through a cycle *in utero*. The inflexion during the 5th month in the curve of the cow, and during the 4th week in the curve of the guinea pig correspond to the maxima in the velocity curves; the flattened segments at about 7 months in the cow, and 32 days in the guinea pig represent the junctions between the first and second cycles.

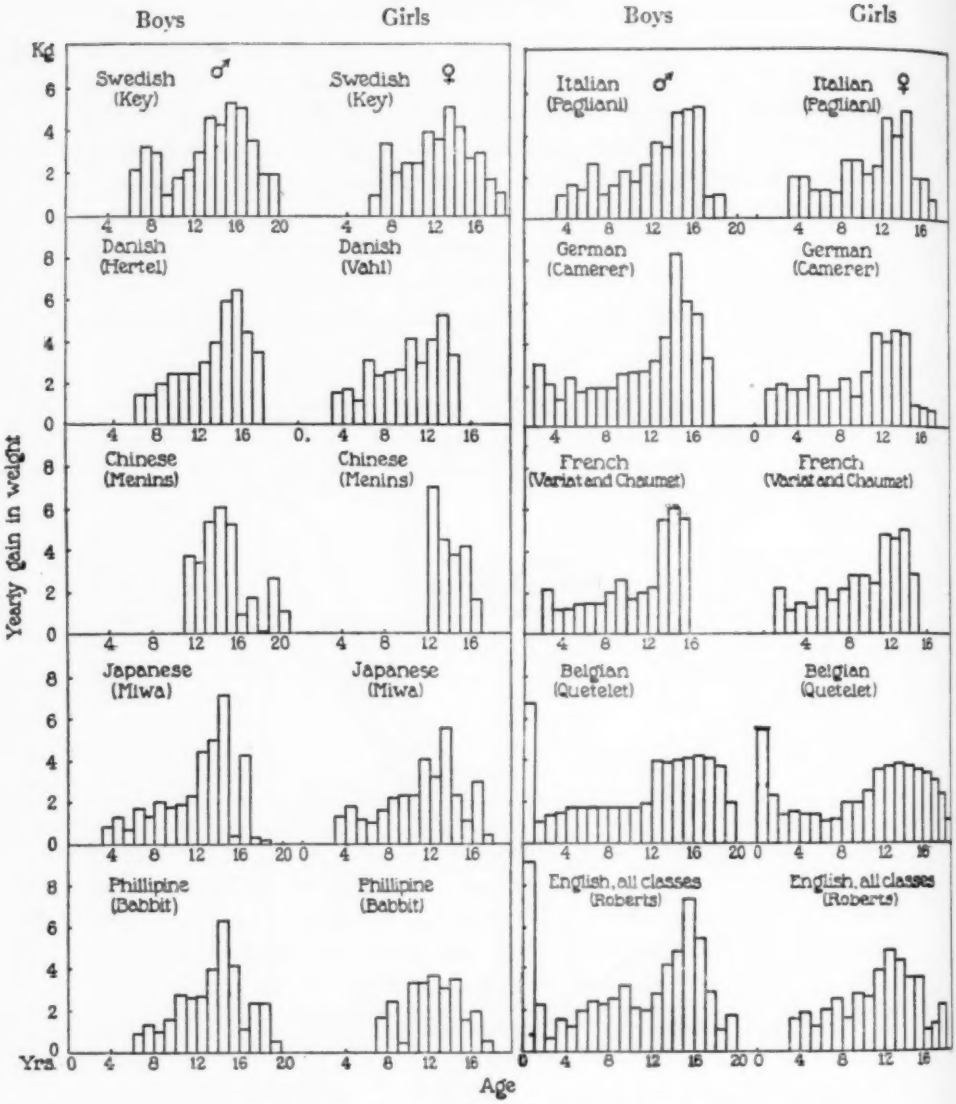


FIG. 7. Velocity curves of growth of European and Asiatic children.

The fact that the age of the adolescent maximum and sexual maturity coincide, does not mean that there is a causative relation between the two. The evidence, with few exceptions, seems to point to the contrary. Stotsenberg[9] found that the rate of growth of normal and castrated rats of the same litter, is practically identical. The curves of the castrated sheep and pig in the figures here presented are not noticeably different from the sexed animals. The curve of the caponized fowl, however, differs from the normal fowl, but this

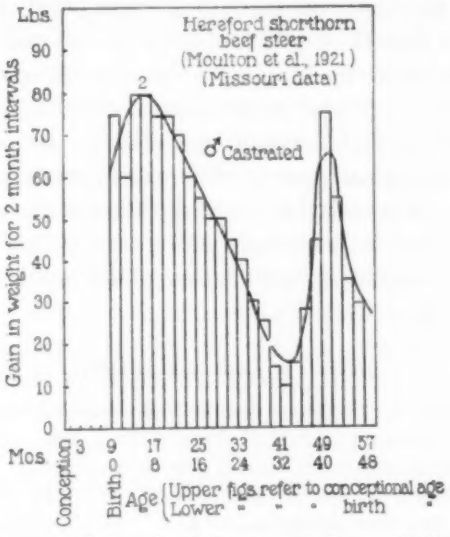


FIG. 8. Velocity curve of growth of the castrated ox. Ordinates and abscissæ have the same meaning as in Fig. 1.

may be due to evident secondary causes. An interesting case is shown by Fig. 8, the curve of the castrated ox. The maximum of the second cycle comes at the same age as in the cow, but the third cycle is either entirely missing, or is very indistinct. Then there is a curious cycle-like rise at about 35 months of age, which is not found in the cow. This apparent exception, however, may be due to some experimental error.

SUMMARY.

1. A method of plotting growth curves is presented which is considered more useful than the usual method in bringing out a number

[9] Stotsenberg, J. M., *Anat. Rec.*, 1909, iii, 233.

of important phenomena such as the equivalence of age in different animals, difference in the shape and duration of corresponding growth cycles in different animals, and also in determinating the age of maxima without resorting to complicated mathematical computations.

2. It is suggested that after the third cycle is past the conceptional age of the maximum of the third cycle may be taken as the age of reference for estimating the equivalent physiological ages in different animals. Before the age of the third cycle, the maxima of the second and first cycles are most conveniently used as points of reference.

3. It is shown that the product of the conceptional age of the maximum of the third cycle by 13, gives a value which is, with the possible exception of man, very near to the normal duration of life of animals under the most favorable conditions of life. In other words, the equivalent physiological ages in different animals bear an approximately constant linear relation to the duration of their growth periods.

4. Attention is called to certain differences in the shape and duration of the corresponding growth cycles in different animals and of the effect of sex on these cycles.

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THE PERMEABILITY OF CELLS FOR OXYGEN AND ITS SIGNIFICANCE FOR THE THEORY OF STIMULATION.

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Although much research has been carried out on the permeability of cells to other substances, relatively little has been done on their permeability to oxygen and carbon dioxide. Yet it is obvious that permeability for these gases is of great theoretical significance in connection with theories of stimulation. Since it is quite certain that oxygen and CO_2 must enter and leave cells during respiration, the question is not one of penetrability, but rather of the rapidity with which they enter or leave the cell. Increased oxidation may result on stimulation of a cell. Is this increase to be explained by assuming increased permeability to oxygen or carbon dioxide? A comparison of the rate of penetration of living and dead cells by these gases will give an answer to the question.

The most significant work in this connection has been done by Jacobs (1920), who finds that carbon dioxide belongs to the group of acids which penetrate a cell with great rapidity. Among acids, CO_2 is comparable to NH_3 among alkalies (Harvey, 1911). Entrance into living cells is just as rapid as entrance into dead cells. Presumably then, we may say that passage of CO_2 from the cell is perfectly free at all times. Is the entrance of oxygen into the cell perfectly free at all times also? A number of lines of evidence bear on this problem and the larger problem whether stimulation brings about an increased permeability of the cell for oxygen.

It is quite possible to show directly by an indicator method, that many kinds of cells are freely permeable to oxygen. I have tried this experiment on epithelial cells of *Chætopterus*, on *Limulus* muscle, on ovarian epithelial cells and the pyloric cæca of the starfish, on the

tentacles of hydroids (*Eudendrium* and *Campanularia*), on gill cilia of *Mytilus*, on liver, muscle, and nerve of the frog. The following experiments will serve as examples of all.

If a frog's muscle is stained in methylene blue and then placed in an atmosphere devoid of oxygen, the blue color disappears. The tissue becomes colorless, an indication that the methylene blue has been reduced to its leuco base. If air is now readmitted to the muscle, the blue color returns *almost immediately* (within 15 seconds). The muscle is perfectly capable of contraction if stimulated electrically, even while the methylene blue is in the reduced or colorless condition. It is not injured by the methylene blue or the removal of the oxygen. The leucomethylene blue simply acts as an indicator of the entrance of oxygen to the cell and gives us unequivocal evidence that the cell is readily permeable for oxygen at all times, even in the resting condition.

If we stain two sartorius muscles in methylene blue, deprive them of oxygen until decolorized, and then kill one muscle with steam in the absence of oxygen, the two, when simultaneously exposed to air, become blue again at approximately the same rate. A dead muscle is, therefore, no more permeable to oxygen than a living muscle. Oxygen enters so readily that no difference is to be seen.

In this experiment, it is convenient to allow the muscle to use up the oxygen, as readily happens. It is sufficient to place a sartorius muscle (carefully removed from the frog, so as to avoid injury, and stained in methylene blue) in Ringer's solution on a slide and cover with a cover-slip. Under the cover, the blue color of the muscle fibers disappears in a short time, since they are denied access to oxygen, and the muscle remains blue only at the edge, where it comes in contact with dissolved oxygen. Oxygen is readmitted by lifting the cover-slip, when the muscle becomes blue throughout.

Use may be made of the avidity of muscles for oxygen to render small amounts of fluids practically oxygen-free. For this purpose, strips of frog muscle (or any muscle tissue) are arranged in the form of a ring on a slide, enclosing a space filled with Ringer's solution or sea water, and containing the tissue upon which the effects of lack of oxygen are to be observed. A cover-slip is now placed over the preparation, with careful exclusion of air bubbles, and we have the

tissue for observation enclosed in a "muscle pen" which quickly frees it of oxygen. This arrangement, which may be easily made, does away with the inconvenience of passing purified hydrogen or nitrogen gas through microscopic gas chambers to remove the oxygen. A control slide with muscle and tissue should be prepared and kept exposed to the air, in order to make sure that non-volatile products, such as lactic acid, diffusing from the muscle are not responsible for effects attributed to lack of oxygen. I have observed no effects which might be due to lactic acid, but it is quite possible that the production of CO_2 may be involved in causing any physiological changes observed. CO_2 accumulation does not affect the color change of the methylene blue, which is entirely dependent on lack of oxygen. The "muscle pen" method is well adapted for class experiments to demonstrate oxygen permeability, as the following example indicates.

Small pieces of the ciliated gill of the mussel, *Mytilus*, are removed and stained in methylene blue. A "muscle pen," using *Limulus* muscle, is then prepared on each of two slides, (A and B), and the *Mytilus* gill tissue placed within in sea water, and covered with a cover-slip. A third slide, (C), is prepared in a similar manner, but is not covered. The cilia in all three preparations are beating vigorously at first, but in the covered preparations (A and B) they gradually slow down and stop, standing straight out from the cells; this occurs in 15 to 30 minutes. The methylene blue color disappears at approximately the same time that the cilia stop beating.[1] In slide C, the blue color does not disappear, and the cilia continue beating vigorously.

Slide B is now steamed for 2 minutes to kill the cilia, and, when cool, the cover-glasses of slides A and B are lifted simultaneously, thus exposing both tissues to the air. The blue color returns in 15 seconds, and at the same rate in both slides. The cilia in the unsteamed slide begin beating again within a minute or two after the blue color has returned. Thus, oxygen enters the cells with practically no resistance.[2]

[1] The relation between stoppage of the cilia and color change of methylene blue depends somewhat on the depth of blue color in the tissue.

[2] This experiment can be performed with practically the same result, if the ciliated cells, stained in methylene blue, are mounted in an Englemann micro-

If kept without oxygen in a "muscle pen" for 6 to 7 hours, the blue color again reappears in 15 seconds after air is admitted, but the cilia do not begin beating so quickly as when the exposure to lack of oxygen is less prolonged. It should be remarked in passing that cilia which have stopped beating from lack of oxygen, will not beat if stimulated with interrupted induced shocks which are sufficiently intense readily to stimulate muscle tissue. It is well known that cilia are remarkably independent of stimulation and in this respect the contractile tissue of cilia behaves quite differently from the contractile tissue of muscle.

Lack of oxygen may be studied with larger whole animals, as follows:

An entire *Chætopterus* is stained in methylene blue and then placed in a glass tube of just the size to fit it. The tube is filled completely with a small amount of sea water, and a rubber stopper carrying a platinum wire (for stimulation) is inserted in each end. The worm gradually uses up the oxygen in the tube. The effect of this on the normal movements of the fan segments, the contraction of muscles and appearance of luminescence on stimulation, and the reduction of methylene blue may be observed.

The first function to disappear is luminescence. The worm may be stimulated to luminescence for 3 to 4 minutes after being placed in the tube, but not after this time. Movement of the fan segments is very slow and weak at the end of 30 minutes, but the methylene blue has not yet been reduced. After $2\frac{1}{2}$ hours, the worms (not observed in the meantime) were colorless, and no spontaneous movement occurred, but contraction of the muscles could be produced on stimulation. If now removed from the glass tube to aerated sea water, the blue color returns in 15 to 20 seconds, and the rhythm of the fan segments is likewise regained. The power of luminescence returns very slowly, however. No luminescence resulted from stimulation $1\frac{1}{2}$ hours after removal to the fresh sea water, but the luminescence had returned in 20 hours.

scopic gas chamber, and nitrogen or (electrolytic) hydrogen gas is passed through the chamber to remove the oxygen. CO_2 passed through the chamber will cause the cilia to stop beating very quickly, but does not decolorize the methylene blue until all the oxygen is driven out.

It may be readily shown by the "muscle pen" method that the living epithelial cells of *Chætopterus* allow oxygen to enter as readily as the dead cells.

I believe we are therefore justified in making the general statement that cells are freely permeable to oxygen at all times. Any theory of stimulation which supposes a sudden increase in permeability to oxygen as a result of stimulation has no basis in fact.[3]

A somewhat similar problem to that of stimulation has to do with the changes occurring in a cell after death. The change occurring on stimulation may be similar in some respects to that resulting from the death of the cell. Thus, a muscle goes into a permanent contracted condition, *rigor mortis*, "death rigor." A luminous cell produces a continual, steady luminescence, the "death glow." The change on stimulation is temporary, that on death is permanent.

On the death of any cell, the chemical reactions run riot. This is especially well seen in some plants, as the potato, false indigo (*Baptisia tinctoria*), or Indian-pipe (*Monotropa uniflora*), which become black on death. Blackening is dependent on oxidation, and may be used as an indication that too rapid oxidation is proceeding within the cells. Why does not this blackening proceed during the life of the cell?

Blackening is due to oxidation of a chromogen to black melanin in the presence of an enzyme, an oxidase. Does this reaction proceed on death of the cell because oxygen can now readily gain entrance, or for some other reason? Perhaps the chromogen and enzyme are kept apart by some mechanism or phase boundary in the living cell which breaks down on the death of the cell.

I have endeavored to gain some light on this problem by subjecting the leaves of the false indigo plant, *Baptisia tinctoria*, to pressures of 1,000 to 1,500 lbs. per square inch of pure oxygen for varying periods of time. This has been accomplished in a small, specially constructed,

[3] This statement must be applied to luminous animals with caution. *Campanularia flexuosa*, a luminous hydroid, possesses cells as freely permeable to oxygen as non-luminous hydroids (*Eudendrium*), but it is impossible to say whether the luminous cells are those which take up the methylene blue. The same statement applies to *Chætopterus*. The photogenic cells of the firefly are large and too easily injured for clear results.

brass chamber connected with a high pressure oxygen tank and gauge.[4] When the pressure chamber was properly connected with lead washers, there was practically no leakage over long periods of time.

It was found that leaves of *Baptisia* would turn black in air saturated with chloroform[5] or ether vapor in less than 5 minutes. Killing the cells in any other way that leaves the enzyme unharmed, gives the same rapid blackening.

Exposure to 1,000 lbs. of pure oxygen for 3 hours does not affect the leaves to the slightest degree. They are green when removed from the chamber and remain green. As 1,000 lbs. of oxygen is about 330 times the pressure of oxygen in the air, we should expect many times more oxygen to dissolve in the cell membranes, although not 330 times as much, for Henry's law cannot be assumed to hold at these high pressures.

As the cells in the leaf which turn black contain chlorophyll (spongy and palisade mesophyll), they must allow oxygen to pass out during photosynthesis,[6] and if blackening depended merely on increased

[4] Caution should be exercised in subjecting dry organic matter to high pressures of oxygen. Explosions have been known to occur as a result of this procedure.

[5] Chloroform was used to kill the cells merely because of its convenience. The chloroform (or ether) has no specific effect in causing the cells to blacken, as blackening results from crushing or freezing the cells (but not from merely keeping the cells at 0°C. for 48 hours). For the same reason, we cannot necessarily suppose that the blackening is due to preponderance of oxidative over reductive processes, as the result of inhibition or destruction of the reducing enzymes by the chloroform. While it is probably true that chloroform destroys the reducing enzymes, but not the oxidizing enzymes, it is well known that plant extracts have reducing power in the absence of oxygen (see Bach, A., *Biochem. Z.*, 1913, lii, 412), and consequently crushing the cell to make an extract does not destroy the reducing enzymes. Nevertheless, crushing a *Baptisia* cell does cause it to blacken.

[6] One might suppose that oxygen never comes in contact with the mesophyll cells, because the stomata might close when the leaf is subjected to high oxygen pressures. While I have never been able to examine the stomata during actual exposure to high oxygen pressures, I have noted that the stomata were open on leaves before and after subjection to high oxygen pressures for times not sufficient to cause leaves to blacken; and also that a leaf cut across and then subjected to high oxygen pressure does not blacken at the cut edge, except in cells actually injured by the cutting. Oxygen under pressure cannot turn the cells black, even when it can come in direct contact with them through the intercellular spaces in the mesophyll leading from the cut edge.

rate of entrance of oxygen, blackening should be rapid at high oxygen pressures. Nevertheless, at 1,000 lbs. pressure, oxygen does not cause blackening as long as the cells are living.

Longer exposure to 1,000 lbs. of oxygen will cause blackening. In 4 hours, the older leaves are slightly darkened and in 7 hours they are completely black when removed from the chamber. This is apparently due to an injury of the cell by the high oxygen pressure, for a leaf removed from the oxygen pressure chamber when slightly blackened along the edges, will turn completely black in the air in the course of some hours. The previous exposure to high oxygen pressure has a progressive injurious effect.

The injurious effect is due to the oxygen, and not to the high pressure, as may be very readily shown by exposing leaves to high hydrogen pressure. Leaves in a pressure chamber filled with hydrogen at 1,800 lbs. of pressure (falling to 1,300 lbs. of pressure during the period of 24 hours) were still green when removed from the chamber, and *remained green for 6 hours afterwards*, when observation was discontinued. They were then subjected to chloroform vapor, to see if the hydrogen had in any way prevented blackening, even of killed cells. It was found that they turned black readily in chloroform vapor in air. Certainly, then, it is not the pressure itself, but the oxygen, which has the injurious action in the high concentration. It would seem that the oxygen under pressure must enter the cell in much higher concentration, but cannot react with the chromogen until some phase boundary or film of separation of chromogen and oxidase is broken down. This film is readily changed on death of the cell.

Finally, I might record the fact that a slice of potato subjected to 2,900 lbs. of air (= 580 lbs. of oxygen), or 1,700 lbs of oxygen pressure for 18 hours was not blackened in the slightest degree, whereas the control in chloroform vapor in air at atmospheric pressure was completely blackened. Again I would not interpret this result as showing that oxygen cannot be forced into living potato cells, but as indicating that it cannot react with the chromogen after it gets in. The boundaries separating chromogen and oxidase appear to be more resistant in the potato than in the false indigo plant.

SUMMARY.

It can be demonstrated by an indicator method that living cells are as freely permeable to oxygen as dead cells, and that sudden admission of oxygen to the cell cannot account for increased oxidation as a result of stimulation. Oxygen penetrates as readily as carbon dioxide among the acids and ammonia among the alkalies.

Exposure of *living* plant cells to *high oxygen pressures* does not initiate certain oxidations (except after some hours), which proceed readily in *dead* plant cells in the *air*. In the light of the preceding statement, about the permeability of cells for oxygen, this is interpreted to mean that more oxygen enters the cell at high pressure, but that the reacting substances (chromogen and oxidase) are kept apart by some phase boundary as long as the cell is alive. Increased oxygen concentration eventually produces injury to the cell.

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THE PERMEABILITY OF LIVING CELLS TO DYES AS AFFECTED BY HYDROGEN ION CONCENTRATION.

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(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, October 9, 1922.)

In order to determine the influence of pH upon the penetration of dyes careful quantitative studies are necessary. In view of the fact that suitable methods are lacking (the only work known to the writer is an excellent paper by Harvey,[1] which, however, is purely qualitative in character) the writer has thought it desirable to develop one, and for this purpose has employed the cells of *Nitella*. These very long multinucleate cells contain a large, central vacuole filled with sap which is surrounded by a delicate layer of protoplasm. If the end of the cell is cut off, and gentle pressure applied, the clear sap flows out, free from protoplasm or chlorophyll granules. If the cell has been previously placed in a dye which has penetrated through the layer of protoplasm into the cell the concentration of the dye in the sap may be measured in a very simple manner. A drop of the sap is taken up into a capillary tube and the color is then compared with the colors of other tubes, of the same diameter, containing different concentrations of the same dye.[2]

For the most part dyes were employed which did not change color on being mixed with cell sap. One of the most useful is a basic dye, brilliant cresyl blue, which was employed in the experiments described in this paper. This dye penetrates rapidly and accumulates within the cell sap so that its concentration becomes much greater than in the outside solution.

[1] Harvey, E. N., *Science*, 1910, xxxii, 565.

[2] When this is done it at once becomes evident that the ordinary method of placing the cell in a dye and observing it under the microscope (without squeezing out the sap) is entirely misleading. In many cases the cell appears to be deeply stained (due to the absorption of the dye by the cell wall or the cell surface) where the sap is only slightly colored. This phenomenon is much more pronounced at certain pH values.

The rate of penetration of cresyl blue increases with increasing pH (the lowest pH employed was 6.5) until, in the neighborhood of pH 10, complications begin to appear which make the determinations doubtful. Thus the rate[3] at pH 7.38 was 5 while at pH 9 it was 350, and at pH 10 it was 910.

As the pH of the cell sap is about 5.6 it is clear that the dye is unable to penetrate rapidly unless the pH outside is decidedly greater than that inside.

Interesting results were obtained in studying the exosmosis of the dye. For this purpose cells were left in 0.002 per cent of cresyl blue at pH 8.98 until the concentration inside reached 0.04 per cent. The cells were then rinsed and placed in buffer solutions (containing no dye). It was found that exosmosis presents a striking contrast to endosmosis as regards the influence of the external pH. Penetration is very rapid at pH 9, while the reverse is true of exosmosis, but at pH 5.9 exosmosis is rapid and penetration is very slow.

Further experiments are in progress which will be described in detail in a later paper.

SUMMARY.

1. An accurate quantitative method of measuring the penetration of dye into the living cell is described.

2. Cresyl blue is unable to penetrate rapidly unless the pH outside the cell is decidedly greater than that inside. The rate of penetration increases with increasing pH.

3. Around pH 9 penetration of the dye is rapid while the reverse is true of exosmosis. At low pH values (5.9) exosmosis is rapid and penetration is very slow.

[3] In this case the rate was taken as a thousand times the reciprocal of the time required (at 20°C.) to raise the concentration in the sap to 0.01 per cent when the concentration of the outside solution was 0.002 per cent. The dye was dissolved in buffer solutions. There was no injury during the period of the experiment.

SOME ASPECTS OF SELECTIVE ABSORPTION.*

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(Received for publication, October 9, 1922.)

The problem of selective absorption presents certain difficulties which are well illustrated by the behavior of potassium. As is well known, potassium may be absorbed in larger quantities than sodium in spite of the fact that sodium greatly predominates in the solution which bathes the cell. Some investigators have attempted to account for this as the result of the formation of insoluble compounds or of selective adsorption, but it is obvious that this cannot furnish a satisfactory explanation for all cases. A much more plausible suggestion is that the potassium combines with some constituent of the cell to form a soluble compound which is unable to pass out through the semipermeable surface of the cell.[1] If sodium forms no such compound, we should expect salts of sodium to diffuse into the cell until they reached the same concentration within and without. This would also be true of potassium but in addition its concentration inside the cell would be increased by the formation of the compound in question.[2]

The writer has had an opportunity to test this suggestion by examining the sap of certain plant cells. Such cells offer especial advantages for the study of this question. It is particularly the potassium in solution which should be considered in this connection. In studying cell sap this presents no difficulty, but in the case of such cells as

* The writer desires to express his great obligation to the trustees of the Elizabeth Thompson Science Fund for a grant to cover the cost of the chemical analysis.

[1] *Cf.* Loeb, J., The dynamics of living matter, New York, 1906, 72. See also Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic and Berlin, 4th edition, 1914, 378, 388. Bayliss, W. M., Principles of general physiology, London, Bombay, Calcutta, and Madras, 1915, 120 ff.

[2] In case this compound dissociated the Donnan effect would play a part.

red blood corpuscles, or of muscle cells, it is impossible to determine how much of the potassium found in the cell is actually in solution.[3]

In the cells studied by the writer the sap is contained in a large central vacuole which is surrounded by a very thin layer of protoplasm, outside of which is the cell wall. In order to reach the vacuole a substance must pass through the outer and inner surfaces of the protoplasm, both of which are to be regarded as semipermeable.

In order to obtain cell sap free from contamination the cells should not be crushed since this sets up reactions by which the composition of the sap is altered, and also allows contamination by substances adhering to the cell surfaces or present in the intercellular spaces. For this reason the writer has studied the sap of certain large cells, which can be obtained without contamination.

The sap chiefly studied is that of the marine alga *Valonia*,[4] which forms large, balloon-shaped cells, each of which may contain several cubic centimeters of cell sap. The writer is under great obligation to Dr. W. J. Crozier, who generously collected the plants at the Bermuda Biological Station of Harvard University and employed the utmost care in extracting and preserving the sap. The plants were removed from the sea water, rinsed with distilled water, wiped dry, punctured with a glass needle and subjected to slight pressure, so that the sap squirted out. The sap was placed in bottles which had been very carefully cleaned; the stoppers were sealed with paraffin or vaseline. The total amount of sap collected was about 300 cc.

The sea water used for comparison was collected at the same time and preserved in the same manner.

The analysis was carried out by Mr. L. M. Van der Pyl under the direction of Professor G. P. Baxter. Preliminary trials showed that it was possible to analyze samples of 10 gm. of sea water with concordant results, which agreed fairly well with the published figures of other workers who have analyzed larger quantities. The analysis of

[3] The fact that an inorganic constituent may be abundantly present in the cell without being in solution is strikingly shown in the case of many plant cells which contain numerous crystals of calcium oxalate while little or no calcium is present in solution.

[4] According to Crozier this is *Valonia macrophysa*. *Cf.* Crozier, W. J., *J. Gen. Physiol.*, 1918–19, i, 581.

sap and of sea water was carried out in precisely the same manner (using samples of about 10 gm. in both cases). Cl was determined as AgCl; SO_4 as $BaSO_4$; Na as NaCl; K as perchlorate.[5] Ca was precipitated as oxalate,[6] ignited, and determined as CaO. Mg, after double precipitation as phosphate,[6] was ignited and determined as pyrophosphate.

It was found that, as stated by Wodehouse,[7] addition of $BaCl_2$ produced no visible precipitate. After addition of $BaCl_2$ the sap was filtered and the residue incinerated. From the resulting weight was subtracted the value obtained by the same procedure (using the same amount of sap) without the addition of $BaCl_2$. The difference was assumed to be $BaSO_4$. Since Wodehouse found that SO_4 penetrates injured cells it is possible that the small amount of SO_4 found in the analysis may be due to the presence of such cells.

Wodehouse, working at Bermuda, found that healthy cells, taken directly from the sea water, gave a test for NO_3 with nitron, while the sea water did not. Tests made by Mr. E. S. Harris on the preserved sap (by the phenoldisulphonic acid method) showed the concentration of NO_3 to be about 0.002 M. The concentration in fresh sap is probably higher than this. Sea water gave no test by this method.

Table I shows the results of the analysis. The value for chlorine in the *Valonia* sap is higher than in sea water. This may be due in part to evaporation during the extraction and subsequent preservation; the sap was contained for the most part in smaller bottles than the sea water and hence might be more affected by evaporation. In this connection it may be noted that the electrical conductivity of the sap was higher than that of the sea water even where both had been kept in glass stoppered bottles for the same length of time (about 3 weeks).

The results differ somewhat from the earlier analyses of Hansen[8] and Meyer.[9] The results of Hansen are only approximations. Those

[5] The potassium was determined according to the method of Baxter, G. P., and Kobayashi, M., *J. Am. Chem. Soc.*, 1917, xxxix, 249; 1920, xlii, 735. Baxter, G. P., and Rupert, F. E., *J. Am. Chem. Soc.*, 1920, xlii, 2046.

[6] *Cf.* Richards, T. W., McCaffrey, C. F., and Bisbee, H., *Proc. Am. Acad. Arts and Sc.*, 1901, xxxvi, 377.

[7] Wodehouse, R. P., *J. Biol. Chem.*, 1917, xxix, 453.

[8] Hansen, A., *Mitt. Zool. Station Neapel.*, 1893, xi, 255.

[9] Meyer, A., *Ber. deutsch. bot. Ges.*, 1891, ix, 77.

TABLE I.

Composition of Valonia Sap and of Bermuda Sea Water.

	Parts per thousand.		Molecular proportions (expressed as per cent of Cl).	
	Bermuda sea water.	Valonia sap.	Bermuda sea water.	Valonia sap.
Cl	19.585 19.600 19.629	21.180 21.186	100.00	100.00
Average.....	19.605	21.183		
Na	10.908 10.929	2.070 2.074	85.87	15.08
Average.....	10.919	2.072		
K	0.457 0.470	20.142 20.143	2.15	86.24
Average.....	0.464	20.143		
Ca	0.456 0.451 0.455 0.451	0.70 0.67	2.05	0.288
Average.....	0.453	0.69		
Mg	1.306 1.308 1.314	Faint tr.	9.74	
Average.....	1.309			
SO_4	3.325 3.328	0.004 0.006	6.26	0.0087
Average.....	3.327	0.005		
Total solids.	45.234 45.222			
Average.....	45.228			
Organic matter.	1.438 1.428			
Average.....	1.433			

of Meyer can not be regarded as accurate; this he recognized and explained on the ground that the amount of cell sap at his disposal was small. No details in regard to analytical methods are given by Hansen or Meyer. Both agree, however, that K is much more abundant than Na in the sap, and Meyer states that Mg, Ca, and SO_4 are less concentrated in the sap than in the sea water, while K is much more concentrated.

The amount of organic matter in the sap is small. It was determined by weighing out samples of approximately 25 cc. into platinum dishes and evaporating, without heat, in a vacuum desiccator. They were then weighed again. The amount of total solids was computed from this figure, which is undoubtedly high, due to the method of evaporation, which may leave as much as 0.5 per cent water included in the crystals. The dishes were then heated over a free flame to char organic matter; they were not allowed to become red hot. The residue was taken up in water, filtered, and evaporated on the steam bath until crystals appeared. The evaporation was completed in a vacuum desiccator and the dishes were weighed. The difference between this weight and that of total solids gives the amount of organic matter. The results are given in Table I.

The table makes it evident that we cannot accept the idea that potassium is held in the cell by entering into combination with an organic compound. It is evident that practically all of the potassium, sodium, and calcium in the cell exists in the form of chlorides.[10]

Evidently there is an excluding mechanism which prevents Na, Mg, Ca, and SO_4 from diffusing into the cell[11] to such an extent as to equalize the concentration within and without. There is also a trapping mechanism which causes KCl to accumulate in the cell until its concentration far exceeds that in the sea water.

[10] The high conductivity of the cell sap shows that these substances must be ionized to about the same extent as in sea water.

[11] Since this mechanism breaks down when the cells are killed it is probable that injured cells absorb more Na, Mg, Ca, and SO_4 than uninjured ones. If injured cells could be wholly excluded when sap is extracted the proportion of these substances might be lower. Wodehouse[7] found that SO_4 penetrates so little into normal cells that the sap gave no visible precipitate with $BaCl_2$; injured cells, on the other hand, gave a test for SO_4.

From a theoretical standpoint, it is clear that these two mechanisms may operate independently.

Discussion may be reserved until the completion of further experiments. It may be remarked, however, that no great stress should be laid on the fact that the conductivity of the sap does not differ greatly from that of sea water. In the case of *Nitella*, whose cell sap may also be obtained without contamination, it is found that the conductivity of the cell sap is very much greater than that of the fresh water in which it grows.

SUMMARY.

1. A mechanism exists in *Valonia* which prevents certain substances (Na, Mg, Ca, SO_4) from reaching as high a concentration inside the cell as in the sea water which surrounds it.

2. A trapping mechanism also exists which causes K to accumulate in the cell in a concentration far in excess of that found in sea water. Practically all the K in the cell exists in the form of KCl.

3. The concentration of Cl does not differ greatly within and without.

4. These facts are not in harmony with present theories regarding the accumulation of K in living cells.

SODIUM CHLORIDE AND SELECTIVE DIFFUSION IN LIVING ORGANISMS.

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I.

Proof that NaCl Acts Similarly to $CaCl_2$ and $LaCl_3$ on the Rate of Diffusion of Acid into the Egg of Fundulus.

1. Each organism and each cell is in dynamic equilibrium with its surroundings, which means that the chemical changes which occur in the cells are, within certain limits, automatically reversed. Although an understanding of the mechanism of this regulation is one of the fundamental problems of physiology, large gaps exist in our knowledge of these regulatory processes. Why is it, for instance, that the blood of bony fishes can maintain a concentration in NaCl far below that of the ocean and far above that of the fresh water in which they may be living? Why is it that the red blood corpuscles[1] or the cells of muscles (and probably cells in general) remain rich in potassium and entirely or almost free from sodium, while the blood is rich in sodium and poor in potassium? Why is it that the waste products, which are formed in one part of the body, diffuse into the blood but do not, as a rule, diffuse into the other cells of the body?

It is the intention of this paper to show that the salts which surround a cell modify the properties of the cell wall in such a way as to accelerate the rate of diffusion of certain ions and retard the rate of diffusion of other ions, and that aside from calcium chloride, sodium chloride is a very important factor in causing such selective diffusion.

Over 20 years ago the writer made the observation that the newly fertilized eggs of the marine fish *Fundulus* which normally develop in sea water, are killed when put into a pure NaCl solution of that

[1] Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1922, liii, 241.

concentration in which NaCl exists in the sea water, while they can live indefinitely in such a solution when a small but definite quantity of a salt with bivalent cation (especially $CaCl_2$) or a still smaller concentration of a salt with a trivalent cation is added.[2] When the eggs are older, they can live for a number of days in a 2.5 M solution of NaCl when the proper amount of $CaCl_2$ is added, while in a pure NaCl solution of that concentration they are killed in a few hours. Since these eggs can develop as well in distilled water as in sea water, it was obvious that the Ca or the other plurivalent cations were not required for any nutritive purpose. In 1905 the writer suggested as an explanation that a pure NaCl solution, if its concentration exceeded a certain limit, made the membrane of the egg more permeable, so that NaCl could diffuse into the egg, killing the embryo; while this increase in permeability was prevented by the presence of a low concentration of Ca.[3] The writer's further experiments on *Fundulus*[4] left little doubt concerning the correctness of this suggestion and it was proven beyond doubt and shown to be a general phenomenon by the beautiful experiments of Osterhout[5] and his collaborators on *Laminaria* and *Nitella*.

While these experiments were correct as far as the rôle of Ca (and other bivalent cations) was concerned, they were incomplete. In these experiments $CaCl_2$ appeared as an antagonist to NaCl, which it was under the conditions of the experiments in which an excess of NaCl was used. The experiments, however, were incomplete inasmuch as they failed to show another equally important fact, namely, that in moderate concentrations NaCl acts exactly like $CaCl_2$ or $LaCl_3$, and that sodium chloride is, perhaps, as important in determining selective diffusion of ions through the cell wall as is $CaCl_2$.

In 1910 Wasteneys and Loeb observed that the marine fish *Fundulus*, which can also live in distilled water, died in 3/10,000 M HNO_3 in less than an hour, while the fish lived indefinitely when the same

[2] Loeb, J., *Am. J. Physiol.*, 1899–1900, iii, 327; 1901–02, vi, 411. *Arch. ges. Physiol.*, 1901–02, lxxxviii, 68.

[3] Loeb, J., *Arch. ges. Physiol.*, 1905, cvii, 252.

[4] Loeb, J., *Science*, 1912, xxxvi, 637; 1916, xliv, 574; *Biochem. Z.*, 1912, xlvii, 127.

[5] Osterhout, W. J. V., Injury, recovery, and death, in relation to conductivity and permeability, Philadelphia and London, 1922.

HNO_3 solution was made up in N/10 NaCl. $CaCl_2$ was even more effective as an antagonist to acid than NaCl.[6] Later the inhibiting effect of salt on the toxic action of acids was observed in the egg of the same fish.[7]

We will show in this paper that NaCl, $CaCl_2$, and $LaCl_3$ act all alike, each one retarding the rate of diffusion of acid through the membrane of *Fundulus* and thereby preventing or retarding the toxic action of the acid on the embryo, with this difference only—that the minimal concentration of the salt in which this inhibiting effect occurs diminishes rapidly with the valency of the cation of the salt.

Two different sets of experiments were made. In one set a certain number of eggs were put into a definite quantity of an acid solution of a definite hydrogen ion concentration and the rate at which acid diffused into the egg was determined by measuring the pH of the solution colorimetrically at different intervals. Since the quantity of acid present in the solution was known in the beginning, it was possible to calculate from the change in pH how much acid had diffused into the egg. In this way the influence of salts on the rate of diffusion of acid into the egg could be measured by following the change of pH of the solution colorimetrically.

In the second set of experiments, twenty eggs were always put into 50 cc. of an acid solution of a definite pH with or without salts, and after a certain number of hours, the percentage of eggs in which the hearts were no longer beating was determined. It may be assumed that the heart of the embryo stops beating when a certain quantity of acid has diffused into the egg, and that this quantity is approximately the same for each heart at the same age of the embryo. There are, however, individual variations in the membranes as a consequence of which the rate of diffusion of acid is not the same for different eggs. Assuming that these individual variations are distributed equally in each group consisting of a larger number of eggs, we may further assume that the percentage of hearts which have stopped beating in each group after a certain time is the expression of the average quantity of diffusion of acid during this time. In this way we gain

[6] Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxiii, 489; 1912, xxxix, 167.
[7] Loeb, J., *J. Biol. Chem.*, 1915, xxiii, 139; 1917, xxxii, 147.

also a measure of the influence of salts on the rate of diffusion of acid through the membrane with the aid of mortality curves.

The eggs used were about 5 days old or older, the heart beat and circulation of the embryos being well developed. The eggs were kept for at least 2 days previous to the experiment in distilled water to free the outside of the membrane from salts. This is absolutely necessary for the study of the influence of salts on the rate of diffusion of acid into the eggs. Twenty eggs which had previously been examined to make sure that their hearts were beating normally were put into an Erlenmeyer flask containing 50 cc. of an acid solution, generally (unless the contrary is stated) HCl of pH 3.7. These acid solutions were made up in either H_2O or salt solution and they were brought to the desired pH with the aid of the proper indicators. It was necessary to compare only such experiments with each other as were made simultaneously to eliminate the influence of temperature. After a certain time the eggs were examined microscopically to determine the percentage of eggs in which the hearts had stopped beating. The pH of the supernatant acid solution was then determined colorimetrically to check the results. It may be said that the colorimetric check was always parallel to the percentage of hearts which had stopped beating. We will first give the results of the influence of salts on the rate of diffusion of acid into the egg by the method of mortality curves.

2. In Fig. 1 are found the mortality curves for HCl of pH 3.7 in the presence of different concentrations of NaCl, $CaCl_2$, and $LaCl_3$. The abscissæ are the concentrations of the salts, the ordinates the percentage of eggs killed by the acid (the stopping of the heart beat by acid becomes rapidly irreversible after a certain time). When all were killed, the value of the ordinate in these mortality curves is 100. The duration of the experiment given in Fig. 1 was about 17 hours. In 17 hours 90 per cent of the eggs were killed by the acid when no salt was present; when salt was present the mortality was less, but the retarding influence of the three different chlorides NaCl, $CaCl_2$, and $LaCl_3$ on the diffusion of acid into the egg increased rapidly with the increasing valency of the cation. Thus to reduce the mortality of eggs in 50 cc. of HCl of pH 3.7 from 90 per cent to 40 per cent, M/500,000 $LaCl_3$ (or $CeCl_3$ or $AlCl_3$) was sufficient, while a con-

centration of between M/8,192 and M/4,096 $CaCl_2$, and of M/64 NaCl was required. In order to overcome completely the toxic effect of HCl of pH 3.7 (*i.e.* to prevent completely the diffusion of acid into the egg) about M/32,000 to M/16,000 $LaCl_3$ (or $CeCl_3$ or $AlCl_3$), about M/1,024 to M/512 $CaCl_2$, and, as we shall see presently, about M/8 to M/4 NaCl were required. The influence of the valency of the

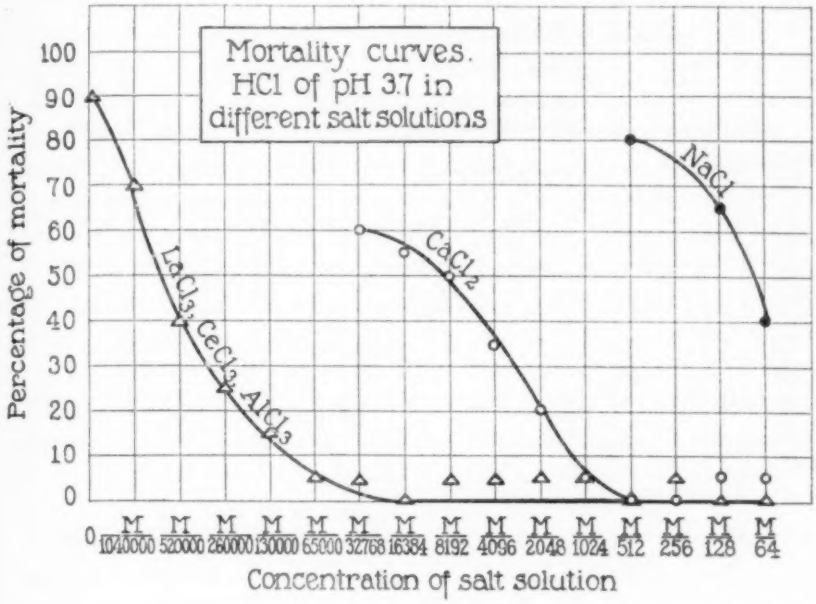

FIG. 1. Inhibiting influence of salts on the toxic effects of HCl solution of pH 3.7. Abscissæ are the concentrations of salts; ordinates, percentage of eggs which died in 18 hours. $LaCl_3$, $CeCl_3$, $AlCl_3$, $CaCl_2$, and NaCl inhibit the toxic effects of acid, but the concentration of salt required diminishes rapidly with the valency of the cation.

cation on the rate of diffusion of HCl of pH 3.7 into the egg is therefore enormous, Ca being about 100 times as effective as Na, and La being at least 40 times as effective as Ca. What interests us here is the fact that NaCl acts qualitatively like $CaCl_2$ and $LaCl_3$ and that only the concentration of salt required differs. The concentration of maximal efficiency of NaCl, namely M/8 to M/4, is that in which it exists in the blood of mammals and many cold blooded vertebrates.

This striking valency effect of the cations suggested a number of additional experiments; namely, first, a comparison of the effect of Na_2SO_4 with that of NaCl. In this case the experiments lasted 3 days. The results are found in Fig. 2. It is obvious that the inhibiting action of Na_2SO_4 on the diffusion of acid is greater than that of NaCl and that therefore the SO_4 ion cannot be said to have an

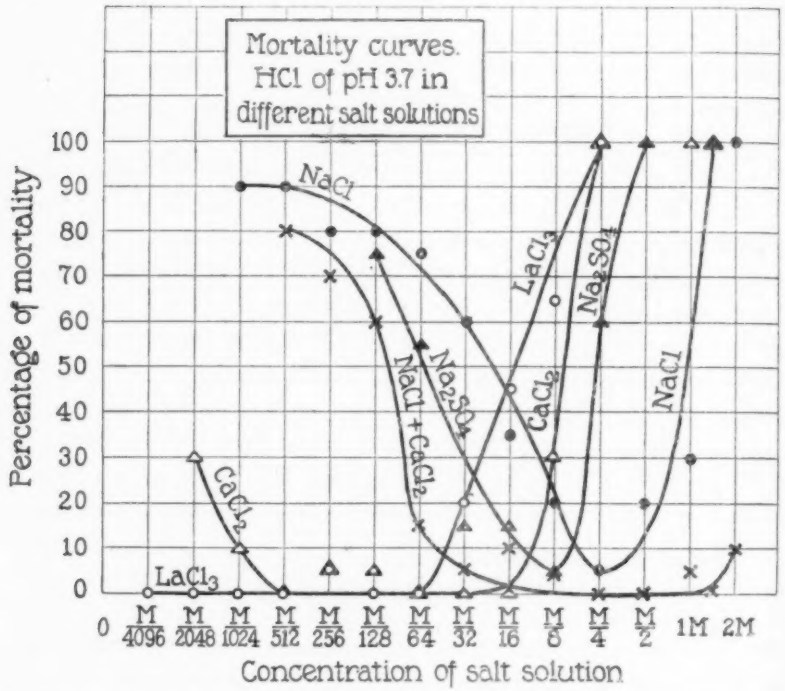

FIG. 2. The inhibiting effect of Na_2SO_4 is greater than that of NaCl but less than that of a balanced solution of NaCl + $CaCl_2$. At higher concentrations this inhibiting effect of salt on the toxic action of acid disappears again.

effect opposite to that of the cation. Fig. 2 gives also a comparison of the inhibiting effect of NaCl alone and of NaCl + $CaCl_2$ (in the proportion of 100 molecules of NaCl to 1.5 molecules of $CaCl_2$). It is obvious that the inhibitory effect of NaCl + $CaCl_2$ on acid is greater than that of NaCl alone, and the effects of NaCl and of $CaCl_2$ seem to be additive. Complete inhibition of the toxic effect of acid (or rather complete inhibition of the diffusion of acid into the egg)

during the 3 days was caused by $LaCl_3$ at a concentration below M/4,096, by $CaCl_2$ at a concentration between M/256 and M/16, by NaCl at M/4, by Na_2SO_4 at M/8, and by a mixture of NaCl + $CaCl_2$ (balanced solution) at M/16.

When the concentration of salts exceeds the concentrations mentioned, they cease to inhibit the diffusion of acid into the egg (Fig. 2). $LaCl_3$ becomes entirely ineffective at a concentration of M/8, NaCl at a concentration of 1 M, $CaCl_2$ at a concentration of M/4, Na_2SO_4 at M/4, while the balanced solution NaCl + $CaCl_2$ remains efficient even at a concentration of 2 M. It is impossible to connect the concentrations at which the salts no longer prevent the diffusion of acid with any purely electrostatic action of ions, and the increase in permeability caused by higher concentrations of salts must be ascribed to an alteration of the membrane due to other than mere electrostatic effects.

This conclusion is corroborated by experiments on the influence of electrolytes other than those thus far mentioned on the rate of diffusion of acid into the egg (Fig. 3). It is obvious that according to the mortality curves in Fig. 3 the inhibiting effect of $MgCl_2$ on the diffusion of acid is very much less than that of $CaCl_2$ and almost approaches that of LiCl or NaCl; and the inhibiting effect of $BaCl_2$ is only slightly greater than that of $MgCl_2$. On the other hand, Mn acts almost as well as Ca. Sr also acts almost like Ca.

These latter facts are, however, only of secondary importance for our purpose which is to show that in the proper concentration NaCl acts exactly like $CaCl_2$ and like $LaCl_3$ on the rate of diffusion of acid through the membrane of the *Fundulus* egg (and, perhaps, through the membranes of cells in general).

3. We will now give the results of the second method of following the diffusion of acid into the eggs colorimetrically. HCl solutions were made up in water or salt solutions to a pH 3.8, and ten eggs, about 5 or 6 days old (with beating hearts) were put into 10 cc. of these HCl solutions of pH 3.8 in a test-tube. 1 drop of methyl orange was added to each solution and the test-tubes were closed with rubber stoppers. From time to time (about every 10 minutes) the test-tubes were slightly agitated to change the solution in immediate contact with the eggs. The color of the indicator made it possible to follow the rate of diffusion of the acid into the egg. It was found

that when salt was present in proper concentration, the color of the solution changed very little or not at all during 20 hours at about 22°C., while when no salt was present the color changed, betraying a gradual diminution of the acid in the solution. By comparing the color of the solution with standardized indicator solutions, the change in pH could be ascertained. Fig. 4 contains some of the results. The ordinates are the pH of the acid solutions while the abscissæ are the times.

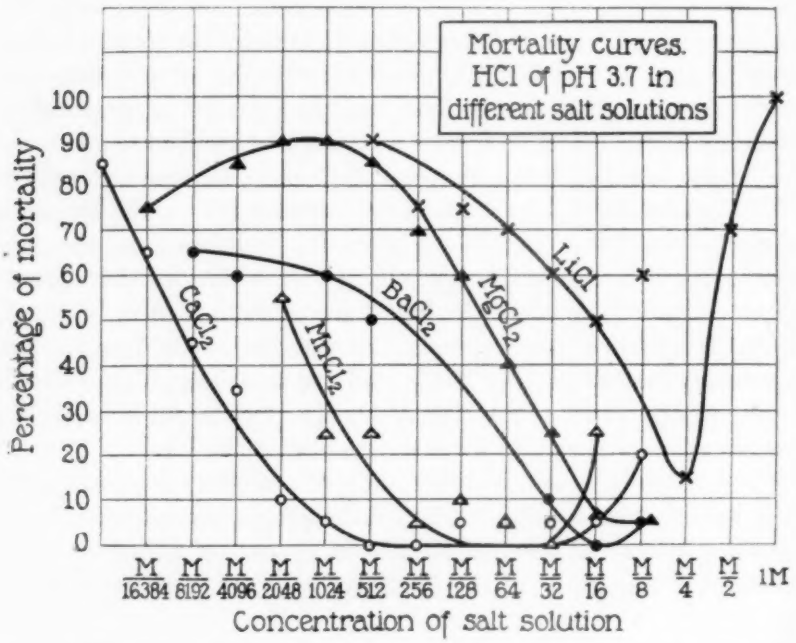

FIG. 3. Proof that the inhibiting effect of $MgCl_2$ and $BaCl_2$ on the rate of diffusion of acid into the egg is less than that of $CaCl_2$.

It is seen from Curve I in Fig. 4 that without salt the pH of the solution rose gradually in 7 hours from 3.8 to pH 4.2. Since there were 10 cc. of solution of HCl present the amount of acid which diffused into the ten eggs can be calculated from the curve. We may omit the calculation, since it is of no special interest in this connection. After 20 hours the pH was over 5.0; *i.e.*, practically all the acid had diffused from the solution into the eggs, and all the embryos were dead

after 20 hours. Curve II in Fig. 4 shows that in M/8, M/4, and M/2 NaCl the pH of the solution did not change during the 20 hours of the experiment; *i.e.*, practically no acid diffused into the eggs and the embryos were all alive after that time. Curve III shows that in

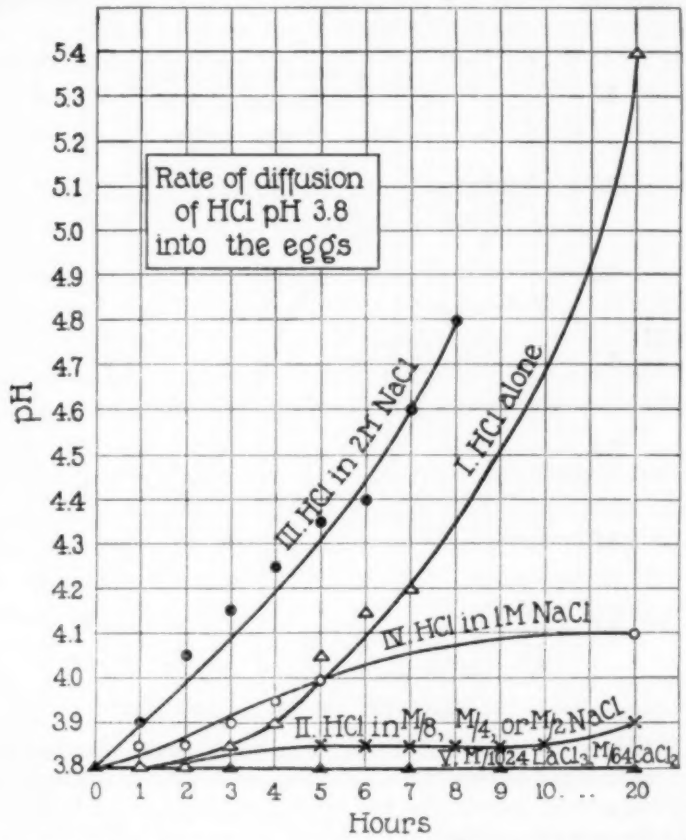

FIG. 4. Influence of salts on rate at which acid diffuses into the egg. Abscissæ are the time in hours, ordinates the pH of the acid solution. The original pH of the acid solution was 3.8.

2 M NaCl the acid diffused even more rapidly into the eggs than without salt and all the embryos were killed rapidly. In 1 M NaCl (Curve IV) the acid diffused first rapidly and later more slowly than without salt; seven of the ten eggs were killed after 20 hours. In M/1,024 $LaCl_3$ and M/64 $CaCl_2$ (Curve V) no color change occurred in the solution

and no acid diffused into the eggs in 24 hours or more and all the embryos remained alive.

In all experiments with mortality curves the pH of the supernatant solution was measured at the end of the experiments. Thus Fig. 5 shows that the curves for the change in pH for NaCl and NaCl +

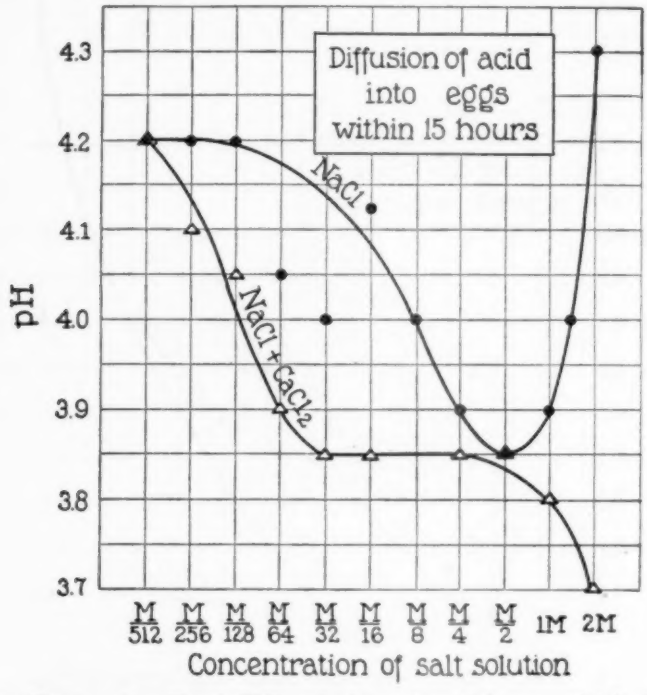

FIG. 5. Change of pH of HCl solution after 15 hours. Original pH 3.7. Abscissæ are the concentrations of NaCl and NaCl+$CaCl_2$; ordinates the pH of the HCl solution after 15 hours. The two curves are similar to the two mortality curves for the same two solutions in Fig. 2.

$CaCl_2$ respectively at the end of the experiment corresponded to the mortality curves for these two solutions in Fig. 2. Where the mortality was lower, the pH curve was also lower, and since the mortality was lower in NaCl + $CaCl_2$ than in NaCl (Fig. 2), we had a right to expect that the same would be true for the pH curves of the two mixtures, which was the case as Fig. 5 shows.

We therefore reach the conclusion that NaCl acts like $CaCl_2$ or $LaCl_3$ in preventing the diffusion of strong acids through the membrane of the egg of *Fundulus*. The only difference is that the concentration in which this salt effect is complete is considerably higher in the case of NaCl than in the case of $CaCl_2$ or $LaCl_3$. The greatest efficiency of NaCl is between M/8 and M/2; *i.e.*, that concentration in which NaCl exists in the sea water and the blood of the majority of animals.

II.

Proof of the Lack of Inhibiting Action of Salts on the Diffusion of Non-Dissociated Weak Acids.

According to Hantzsch, fatty acids, like acetic acid, occur in two tautomeric forms, true acids (which always undergo complete electrolytic dissociation), and pseudo-acids which are not dissociable. There exists an equilibrium between the two forms which upon the addition of a salt like Na acetate shifts in favor of the non-dissociable pseudo-acid form.

As long as the concentration of acid is not too high, so that there is not too high a concentration of pseudo-acid, acetic acid acts much like HCl in the egg of *Fundulus* and the rate of diffusion of acetic acid into the egg of *Fundulus* is retarded by the presence of salt. If, however, much pseudo-acid is present, the egg is no longer protected by salts.

Fig. 6 shows the mortality curve of *Fundulus* eggs in acetic acid, HCl, H_2SO_4, and H_3PO_4, plotted over the pH as abscissæ with the percentage of mortality (in 14 hours) as ordinates. It is obvious that acetic acid of the same pH is a little more toxic than HCl and this difference may be ascribed to undissociated acid. The fact that fatty acids are more efficient physiologically than HCl of the same hydrogen ion concentration has been known for a long time. Richards[8] observed it as far as the minimal concentration of acid for sour taste is concerned, the writer observed it in regard to the effect of the velocity of swelling of muscle in acid,[9] and later in a most striking

[8] Richards, T. W., *Am. Chem. J.*, 1898, xx, 121.

[9] Loeb, J., *Arch. ges. Physiol.*, 1898, lxxi, 457.

way in his experiments on membrane formation in sea urchin eggs in which CO_2 and fatty acids were very efficient and strong mineral acids inefficient.[10] He explained the phenomenon on the assumption of the greater diffusibility of the non-dissociated acid molecule.[10] As soon as the non-dissociated acid molecule gets through the cell wall, it will be transformed from the non-dissociable pseudo-acid form into the dissociable-acid form, producing the acid effect, *e.g.*, sour taste, swelling of muscle (due to formation of a dissociable pro-

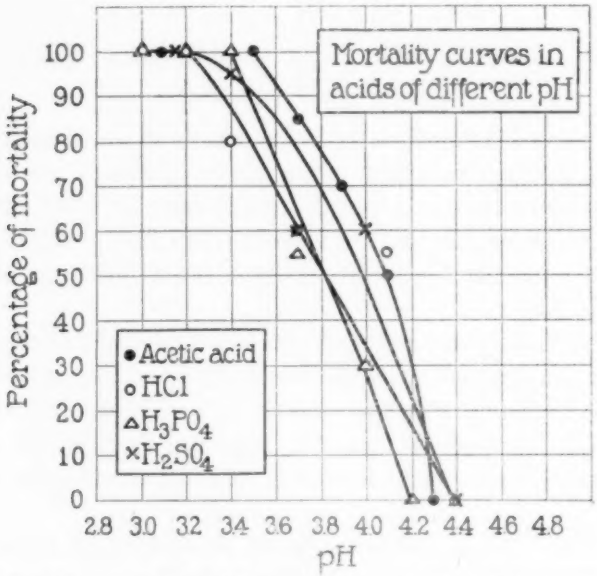

FIG. 6. Mortality curves for different acids. Acetic acid is more toxic than HCl of the same pH, especially at lower pH where the quantity of undissociated acetic acid becomes greater.

tein-acid salt and the establishment of a Donnan equilibrium), or membrane formation in the case of the sea urchin egg. Jacobs more recently demonstrated beautifully the greater diffusibility of non-dissociated acid through living membranes.[11]

It is possible to retard the diffusion of HCl and of acetic acid of pH 3.8 by NaCl, but this inhibitory effect of NaCl is much less in the

[10] Loeb, J., Artificial parthenogenesis and fertilization, Chicago, 1913.

[11] Jacobs, M. H., *Am. J. Physiol.*, 1920, li, 321; liii, 457.

case of the diffusion of acetic acid than in the case of HCl. This is shown by the mortality curves in Fig. 7. Thus when HCl of pH 3.8 was dissolved in M/32 NaCl, only 20 per cent of the embryos had been killed after 12 hours, while when acetic acid of pH 3.8 was dissolved in M/32 NaCl, 40 per cent were killed. To keep all the eggs alive for 12 hours in acid of pH 3.8, M/16 NaCl was required in the case

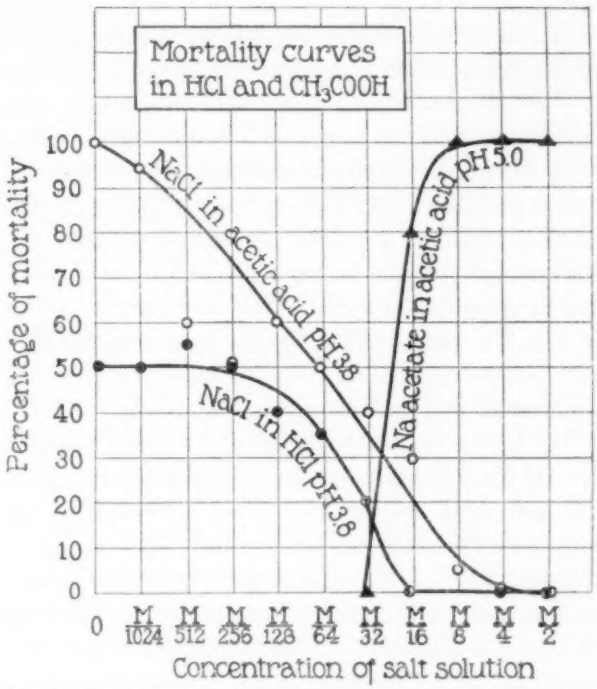

FIG. 7. Showing that NaCl inhibits the toxic action of HCl and acetic acid of pH 3.8, while Na acetate renders acetic acid of pH 5.0 already toxic when the concentration of this salt exceeds M/32. This toxicity is due to the non-dissociated acetic acid which is the greater the higher the concentration of Na acetate.

of HCl and M/4 NaCl in the case of acetic acid. This could be explained on the assumption that the NaCl prevents the diffusion of the dissociated acid in both cases but does not prevent the diffusion of the non-dissociated molecules of acetic acid to the same degree, if at all. HCl solution of pH 3.8 has less than N/8,000 titrable acid, while an acetic acid solution of pH 3.8 has about ten times as much titrable acid, nine-tenths of which is not dissociated.

To test further the idea that salts only inhibit the diffusion of dissociated but not of non-dissociated acid, experiments were made with mixtures of acetic acid of pH 5.0 and Na acetate. Acetic acid of pH 5.0 is harmless for the egg, as Fig. 6 shows. It was ascertained that M/32 to M/4 solutions of Na acetate were also harmless for the egg of *Fundulus* at pH 5.8. Acetic acid of pH 5.0, however, kills all the eggs rapidly if it is made up in Na acetate of M/16 or above. The reason is that to give a mixture of M/16 Na acetate and acetic acid a pH of 5.0, a concentration of between M/64 and M/32 acetic acid is required. The diffusion of non-dissociated acetic acid into the eggs killed the embryos at as low a pH as 5.0, at which strong acids like HCl or H_2SO_4 are quite harmless for the egg of *Fundulus*.

The fact that salts like NaCl can prevent the diffusion of dissociated acid but cannot prevent the diffusion of non-dissociated acid into the egg of *Fundulus*, seemed of such physiological importance as to make further proof more desirable. This was done in the experiments recorded in Fig. 8. The abscissæ in this figure are the pH while the ordinates are the percentage of embryos killed by the acid in about 14 hours. Curve II in Fig. 8 shows that when acetic acid is made up in M/1,024 $LaCl_3$, the eggs are not killed until the pH is below 3.5; while Curve III shows that in acetic acid alone the eggs are already killed when the pH is below 4.3. This difference is considerable and shows that $LaCl_3$ has a great retarding effect on the diffusion of dissociated acetic acid. This retarding effect of $LaCl_3$ on acetic acid is, however, less than on HCl, since Curve I shows that $LaCl_3$ inhibits the effect of the strong acid HCl much more effectively than it does the weak acetic acid. The difference is due to the fact that the $LaCl_3$ retards the diffusion of dissociated acid, HCl or acetic, but not of the non-dissociated pseudo-acid, CH_3COOH. This is made certain by Curves IV and V. Curve IV shows that in a mixture of acetic acid and M/16 Na acetate the eggs were killed at a pH of 5.1, and Curve V shows that this mixture is only a little less toxic in the presence of M/1,024 $LaCl_3$. The slight antagonistic effect revealed by a comparison of Curves IV and V is due to the inhibitory effect of $LaCl_3$ on the diffusion of the dissociated acid into the egg. The experiment was repeated with M/64 $CaCl_2$ instead of $LaCl_3$ and the result was the same.

By mixing acetic acid with M/16 Na acetate the equilibrium is shifted in favor of non-dissociated acetic acid, and M/64 acetic acid is required to bring a solution of M/16 Na acetate to a pH of 5.1. The high concentration of undissociated acetic acid killed the eggs and the experiments all proved that the rate of diffusion of this

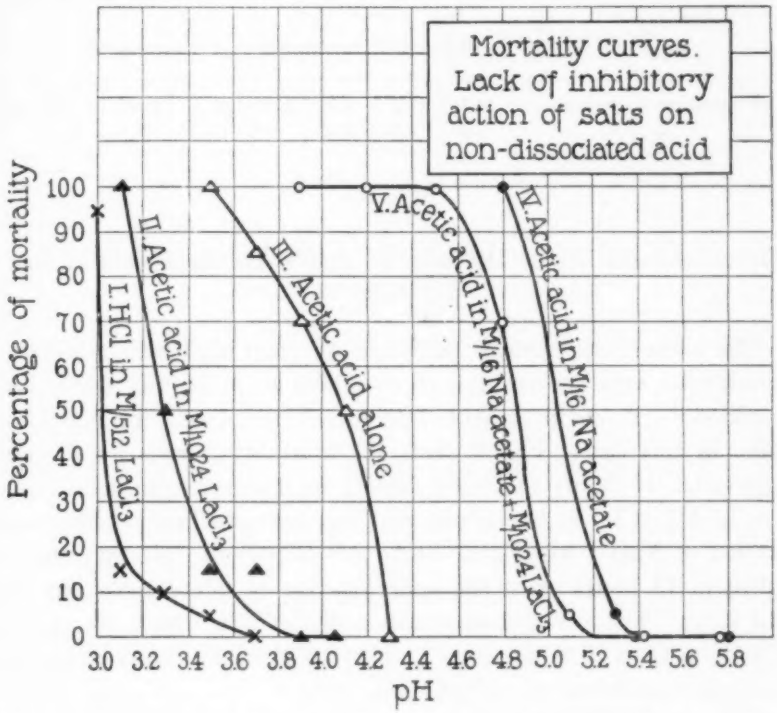

FIG. 8. Proving that $LaCl_3$ only inhibits the diffusion of dissociated acid into the egg but does not inhibit the diffusion of the non-dissociated acetic acid.

non-dissociated acid cannot be inhibited at all or only to a negligible extent by the presence of salt.

We therefore see that while the proper concentrations of salts like $LaCl_3$, $CaCl_2$, and NaCl prevent the diffusion of electrolytically dissociated acid through the membranes of *Fundulus*, they will not prevent the diffusion of non-dissociated acetic acid—the so called pseudo-acid CH_3COOH. It is needless to say that this difference in the

action of salts on dissociated and non-dissociated (or pseudo-) acids must be of importance for the selective diffusion of acids through the kidneys.

In the following table is shown the concentration of acetic acid required to bring a M/16 solution of Na acetate to different pH. The pH was practically the same when M/1,024 $LaCl_3$ was in the solution.

M/16 Na Acetate in Different Concentrations of Acetic Acid.

Concentration of acetic acid..	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256
pH.....	3.6	4.0	4.2	4.5	4.8	5.1	5.4	5.8

III.

The Acceleration of the Diffusion of Alkali into the Fundulus Egg by Salts.

The writer had already published the fact that either NaOH or ethylamine kills the embryo of *Fundulus* more rapidly when a salt is added.[12] It was intended to find out whether $CaCl_2$ and NaCl acted alike in this case. Fig. 9 gives the mortality curves for *Fundulus* eggs after 15 hours in N/250 NaOH in different concentrations of $CaCl_2$ or NaCl. It is obvious that the eggs can live in this concentration of NaOH when little or no salt is present, but that they are killed in 15 hours when the concentration of salt exceeds a certain low limit. The lowest concentration of $CaCl_2$ in which the eggs can live more than 15 hours at 24°C. is M/1,024, while the corresponding minimal concentration of NaCl is M/256.

Fig. 10 was intended to compare the effect of Na_2SO_4 and NaCl for a N/300 solution of NaOH. There is little difference between NaCl and Na_2SO_4, and less between NaCl and $BaCl_2$ than between NaCl and $CaCl_2$ in Fig. 9. In all cases there is an acceleration of the diffusion of alkali into the cell due to salt.

What interests us is the fact that NaCl and $CaCl_2$ act alike on the rate of diffusion of alkali into the egg with this difference only that $CaCl_2$ is more effective than NaCl in accelerating the rate of diffusion of NaOH through the membrane of the egg of *Fundulus*.

[12] Loeb, J., *J. Biol. Chem.*, 1917, xxxii, 147.

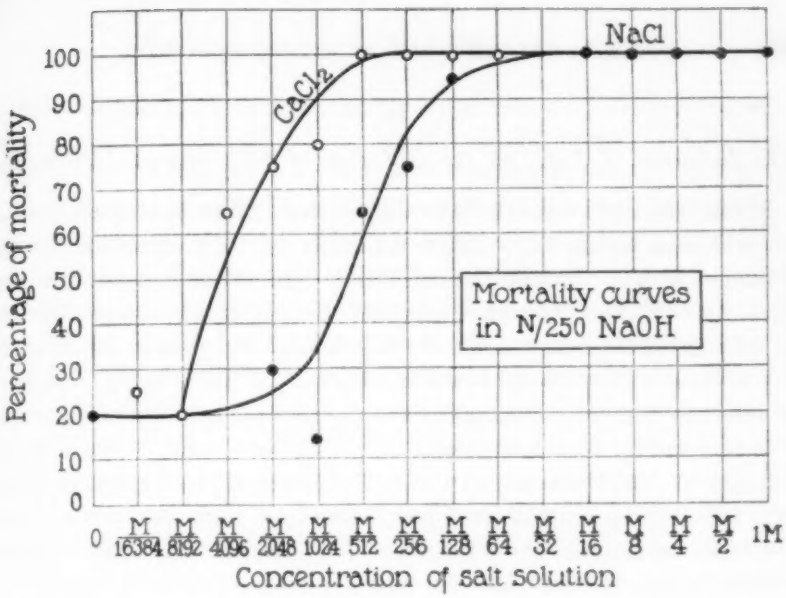

FIG. 9. $CaCl_2$ and NaCl accelerate the rate of diffusion of NaOH into the egg, $CaCl_2$ being more effective than NaCl.

FIG. 10. Accelerating influence of different salts on the diffusion of NaOH into the egg.

IV.

The Influence of NaCl on the Diffusion of KCl through Membranes.

While NaCl and $CaCl_2$ behave alike in their influence on the diffusion of acid and alkali, they differ strikingly in their influence on the diffusion of KCl through the cell wall. The writer is inclined to believe that this fact may have an effect in maintaining the equilibrium of KCl inside the cells. Cells are rich in KCl and poor in NaCl, while the reverse is true for the blood serum and the tissue fluids. In 1911 Wasteneys and the writer made the observation that *Fundulus* could live indefinitely in a low concentration of KCl, while when a slight amount of NaCl was added the fish died rapidly; when more NaCl was added the fish again lived indefinitely. A slight amount of NaCl therefore accelerated the diffusion of KCl into the egg while a higher concentration of NaCl had the opposite effect, retarding the rate of diffusion of KCl into the fish.[13]

Similar results were observed by Cattell and the writer on the egg of *Fundulus*.[14] A concentration of KCl which alone was not able to cause the heart to stop beating was rendered efficient when a little NaCl was added, while with the addition of more NaCl the diffusion of KCl into the egg was blocked again.

When embryos whose hearts had been caused to stop beating in a KCl solution were put into a NaCl solution (or solutions of certain other salts), the heart beat was resumed again after some time; but when the eggs poisoned by KCl were put into distilled water or a solution of a non-electrolyte, such as cane-sugar or glucose or glycerol or urea, the hearts would not recover. These embryos have the peculiarity that they can survive for as long as a week or more without heart beat. It was found that such eggs which had failed to recover from the KCl in distilled water or sugar solutions for several days would recover promptly when put into the solution of the proper salts. All these experiments proved that the diffusion of KCl through the membrane of the egg was only possible when the egg was in a solution of a sodium salt or when the membrane contained some such

[13] Loeb J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxi, 450; xxxii, 155.

[14] Loeb, J., and Cattell, McK., *J. Biol. Chem.*, 1915, xxiii, 41. Loeb, J., *J. Biol. Chem.*, 1916, xxvii, 339, 353, \$63.

salts. This salt effect required for the diffusion of KCl through the membrane increased for sodium salts with the valency of the anion. The reader is referred for further details to the previous publications.[18]

In these older experiments one question was left undecided; namely, whether or not $CaCl_2$ acts like NaCl on the rate of diffusion of KCl through the membrane of the egg. In order to obtain a definite answer a special method must be used. Eggs of *Fundulus* in which the circulation was developed were taken from sea water and put for 48 hours into a M/2 KCl solution of pH about 5.8 at a temperature of about 24°C. The purpose was to fill the eggs with an excessive dose of KCl so that the recovery should not be too quick. This precaution had been neglected in the writer's previous experiments and as a consequence recoveries occurred for which the outside solution was not always responsible. It was assumed that the recovery, *i.e.* the resumption of the heart beat, occurred when the excess of KCl had diffused out of the egg. The eggs thus poisoned with KCl were then transferred into distilled water for 2 days to remove all salts (adhering to the outer surface of the egg membrane). None of the hearts recovered in the distilled water. The eggs were then put into different concentrations of different salts all of a pH of 5.8 (*i.e.* the pH of the distilled water) and examined after certain intervals. Twenty eggs were always put into 50 cc. of the salt solution. Fig. 11 gives the curves for the recovery of eggs in 3 days in solutions of NaCl, $CaCl_2$, and $LaCl_3$. The abscissæ are the concentrations of the salt solutions, the ordinates the percentage of eggs that had recovered. It is obvious that recovery occurred rapidly in NaCl, while the recovery was very slow in $CaCl_2$ and while practically no recovery occurred in $LaCl_3$. It was not due to any injurious action of $CaCl_2$ that the eggs did not recover since it was found in control experiments that eggs which had failed to recover after 2 or 3 days in, *e.g.*, a M/64 solution of $CaCl_2$, recovered in less than a day when put into a solution of M/64 $Na_4Fe(CN)_6$ or some other sodium salt.

Since in sea water and in blood NaCl is always accompanied by $CaCl_2$ in the proportion of approximately 100 molecules of NaCl to 1.5 molecules of $CaCl_2$, it seemed of importance to determine the influence of different concentrations of such mixtures of NaCl + $CaCl_2$ on the rate of diffusion of KCl through the membrane. These experiments

yielded the interesting results that the balanced solution of NaCl + $CaCl_2$ accelerated the diffusion most at a concentration of M/4 and M/2 while at higher concentrations the influence became less (Curve I, Fig. 12). This was not due to any injury to the egg but corresponded to the earlier results that when the concentration of NaCl exceeds a certain limit it retards the diffusion of KCl through

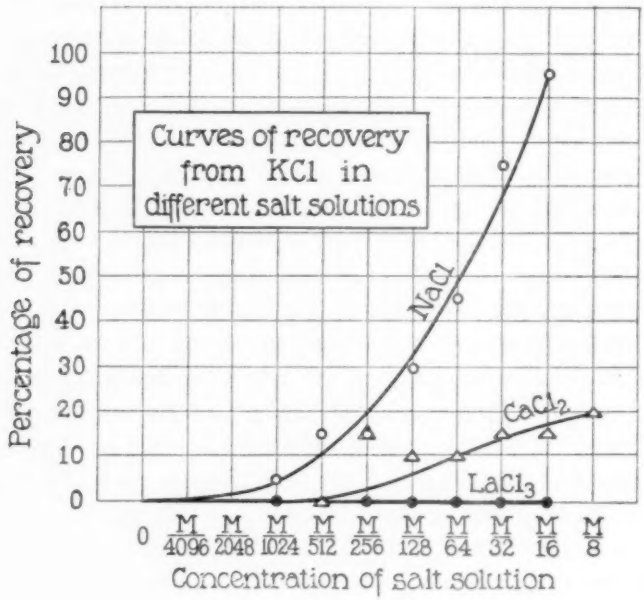

FIG. 11. Influence of various salts on the rate of recovery of eggs from an excess of KCl. Abscissæ are the concentrations of salts; ordinates, percentage of eggs which have recovered from KCl. Recovery is most rapid in NaCl, less rapid in $CaCl_2$, and least rapid in $LaCl_3$. pH 5.8.

the membrane. That the higher concentration of NaCl + $CaCl_2$ only retarded the rate of diffusion of KCl without injuring the egg follows from the fact that 24 hours later more eggs had recovered in these higher concentrations than had after 20 hours (Curve II, Fig. 12).

It had already been noticed in the older experiments that the Na salts caused the recovery of the eggs the more promptly the higher

the valency of the anion of the salt. Since in these experiments no attention had been given to the pH of the solution, it was necessary to repeat the experiments. In the new experiments the salt solutions always were made up for the same pH; namely, 5.8. It is obvious that the recovery is the most rapid in $Na_4Fe(CN)_6$, next in Na_2SO_4, and next in NaCl, while it is slowest in $MgCl_2$ (Fig. 13).

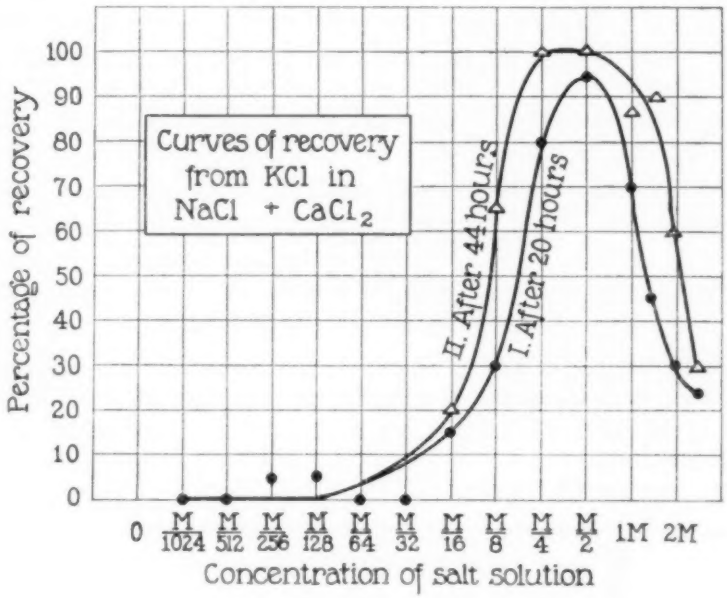

FIG. 12. Influence of concentration of NaCl + $CaCl_2$ (balanced solution) on recovery of eggs from KCl poisoning, showing that when the concentration of salt increases from M/128 to M/2 the rate of recovery increases, while after that the rate of recovery diminishes with further increase in concentration.

It may incidentally be stated that the recovery as a rule was a little quicker in $MgCl_2$ or $BaCl_2$ than in $CaCl_2$, while $MnCl_2$ and $SrCl_2$ acted very much like $CaCl_2$. In LiCl and NH_4Cl the recovery was decidedly slower than in NaCl (Fig. 14).

Entirely similar results as those described for the rate of diffusion of KCl were obtained in regard to the influence of these salts on the rate of diffusion of Rb through the membrane of the egg of *Fundulus*.

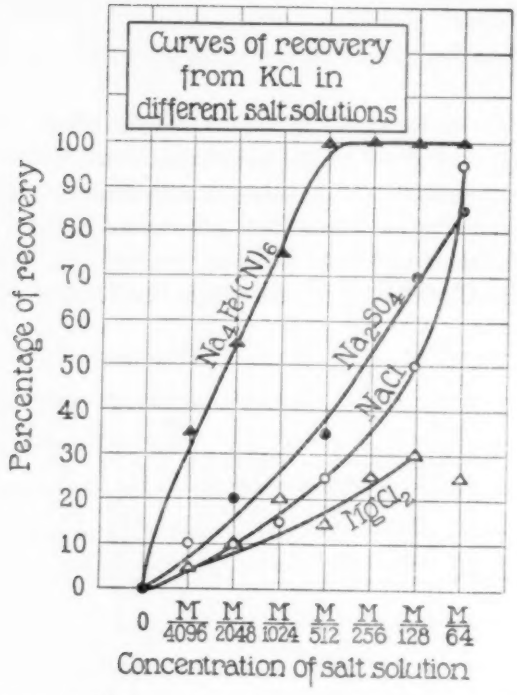

FIG. 13. Influence of different salts on the rate of recovery. The rate increases for Na salts with the increase in valency of the anion. pH 5.8.

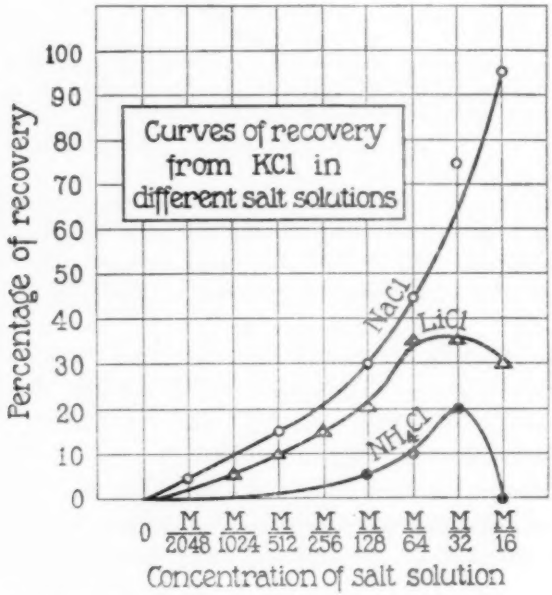

FIG. 14. Influence of NaCl, LiCl, and NH_4Cl on the rate of recovery from KCl. Influence of NaCl is greater than that of LiCl or NH_4Cl.

THEORETICAL REMARKS.

It has been customary to explain phenomena of selective diffusion through membranes as well as differences in the relative concentration of ions between cells and blood by the assumption that the diffusion depends on the lipoids of the cells and that only substances that are soluble in these lipoids can diffuse through the cell walls. Thus K was considered lipoid-soluble, and Na and Ca were not considered lipoid-soluble. These ideas are purely speculative and the best that can be said for them is that they are not contradicted by the facts and that possibly at some future date these guesses may turn out to have been correct. The facts enumerated in this paper point, however, to a definite influence of NaCl and $CaCl_2$ on selective diffusion and these facts exist and are of importance regardless of the ultimate explanation of the mechanism by which salts influence diffusion through the membrane of cells.

We understand why Na and Ca cannot diffuse into the cells of our body, since such a diffusion is rendered impossible by both the $CaCl_2$ and the NaCl present in blood, which seem to render the membrane of most kinds of cells impermeable for Na as well as Ca. We see, on the other hand, that the NaCl must favor the diffusion of KCl into the membrane, and this favorable effect is not counteracted by the Ca, as Fig. 12 shows. If it could be shown that NaCl + $CaCl_2$ favor also the rate of diffusion of Cl ions through the membrane, it would follow that KCl must accumulate in the cells until the molar concentration of Cl ions in the cells equals the Cl ions in the blood or tissue fluid. While this may be the case, the writer does not wish it understood that he considers this influence of NaCl upon the diffusion of KCl through the membrane as the sole cause of the unequal distribution of K and Na in cells and blood. That such a suggestion would be premature is indicated by the work of Loeb, Atchley, and Palmer.[15] It is certain, however, that the effect of NaCl + $CaCl_2$ upon the rate of diffusion of KCl through the membrane must influence the ease with which this ionic equilibrium between blood and cells is maintained.

[15] Loeb, R. F., Atchley, D. W., and Palmer, W. W., *J. Gen. Physiol.*, 1921–22, iv, 591.

The fact that salts accelerate the rate of dissociated alkalies and retard the rate of diffusion of dissociated acids, while they have no retarding influence on the rate of diffusion of non-dissociated acid (and, perhaps, also non-dissociated alkali) has probably some bearing on secretions and certainly an important bearing on the use of drugs and disinfectants.[16] It is also possible that the lack of inhibitory action of salts on the rate of diffusion of non-dissociated acids may ultimately furnish a clew for the understanding of the mechanism by which salts modify the rate of diffusion of ions through the membrane.

Finally the writer calls attention to the fact that salts accelerate the rate of diffusion of acids through collodion membranes[17] while they have the opposite effect on the rate of diffusion of acids through the membranes of *Fundulus*. This must be due to the fact that the *Fundulus* membrane or the membranes of cells in general differ from collodion membranes in a chemical or physical way.

It may also be stated that acid diffused more rapidly into eggs which had been killed by keeping them for 5 minutes at a temperature of 80°C., but that the rate of diffusion of acid into such eggs is also retarded by salts.

SUMMARY AND CONCLUSIONS.

1. It is shown that NaCl acts like $CaCl_2$ or $LaCl_3$ in preventing the diffusion of strong acids through the membrane of the egg of *Fundulus* with this difference only that a M/8 solution of NaCl acts like a M/1,000 solution of $CaCl_2$ and like a M/30,000 solution of $LaCl_3$.

2. It is shown that these salts inhibit the diffusion of non-dissociated weak acid through the membrane of the *Fundulus* egg but slightly if at all.

3. Both NaCl and $CaCl_2$ accelerate the diffusion of dissociated strong alkali through the egg membrane of *Fundulus* and $CaCl_2$ is more efficient in this respect than NaCl.

4. It is shown that in moderate concentrations NaCl accelerates the rate of diffusion of KCl through the membrane of the egg of *Fundulus* while $CaCl_2$ does not.

[16] Michaelis, L., and Dernby, K. G., *Z. Immunitätsforsch., Orig.*, 1922, xxxiv, 194.
[17] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 255.

THE INFLUENCE OF SALTS ON THE RATE OF DIFFUSION OF ACID THROUGH COLLODION MEMBRANES.

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When a solution of a salt, *e.g.*, $CeCl_3$ or $CaCl_2$ or NaCl, in HCl, with a pH of 3.0, is separated by a collodion membrane from a pure solution of HCl of the same pH, salt will diffuse out until the solution on both sides of the membrane is identical. Since the concentration of HCl measured by the hydrogen electrode is the same on opposite sides of the membrane at the beginning of the experiment, one would expect that no change in the pH should occur during the experiment. The writer found, however, that at first acid is rapidly driven by the salt to the other side of the membrane, so that at first the concentration of the acid is diminished on the side of the salt solution and increased on that side of the membrane where the solution was originally free from salt.[1] This occurs, of course, only during the first hours of the experiment. When the concentration of the salt has become the same on the opposite sides of the membrane, the pH also becomes the same again on both sides of the membrane.

The following experiments may serve as an illustration. Collodion bags, treated with gelatin, of about 50 cc. contents, were filled with a M/256 solution of a salt made up to pH 3.0 through the addition of HCl. These bags dipped into beakers filled with 350 cc. of HCl of pH 3.0 (about N/1,000) but without salt. After different intervals the experiment was terminated and the pH inside and outside was determined. It was found that after an hour the pH inside had increased considerably (*i.e.* the concentration of hydrogen ions in the salt solution had diminished), while outside the change of pH was small on account of the fact that the volume of solution was seven times as great as inside the bag. Table I gives some of the results. In Experiments 1 to 4 the collodion membrane was coated with gelatin, while in Experi-

[1] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 213.

ment 5 the membrane was not treated. The phenomenon is observed in both cases. The initial concentration of the salt inside the bag was always M/256, but, of course, diminished during the experiment.

TABLE I.

Change of Hydrogen Ion Concentration on Opposite Sides of the Membrane during Period of Diffusion of Salts.

Time, *hrs.*		0	1	2	3	5	6	8	22
1. M/256 $CeCl_3$.	pH inside	3.01	3.29	3.29	3.32	3.17	3.20	3.16	3.04
	pH outside	2.97	2.97	2.95	2.95	2.97	2.98	2.97	2.99
	Difference	0.04	0.32	0.34	0.37	0.20	0.22	0.19	0.05
2. M/256 $CaCl_2$.	pH inside	2.99	3.26	3.26	3.21	3.20	3.21	3.10	3.02
	pH outside	2.97	2.97	2.97	2.96	2.98	2.98	2.98	2.99
	Difference	0.02	0.29	0.29	0.25	0.22	0.23	0.12	0.03
3. M/256 NaCl.	pH inside	2.98	3.13	3.12	3.09	3.05	3.05	3.00	
	pH outside	2.98	2.98	2.99	2.98	2.99	3.00	2.99	
	Difference	0	0.15	0.13	0.11	0.06	0.05	0.01	
4. M/256 Na_2SO_4.	pH inside	3.06	3.08	3.01	3.01	3.01	3.01	3.05	
	pH outside	2.98	3.01	3.00	3.01	3.00	3.02	3.03	
	Difference	0.08	0.07	0.01	0	0.01	0.01	0.02	
5. M/256 $CaCl_2$. (Membrane not coated with gelatin.)	pH inside	2.98	3.11	3.11	3.08	3.04	3.04	3.02	
	pH outside	2.98	2.95	2.97	2.99	2.97	2.97	2.99	
	Difference	0	0.16	0.14	0.09	0.07	0.07	0.03	

These experiments show, first, that the expulsion of acid from the salt solution into the solution free from salt during the first hours of the experiment is in the case of chlorides the more considerable the higher the valency of the cation; in other words, it increases with the concentration of Cl ions in the solution. Thus after 3 hours the difference in pH inside and outside is 0.37 for $CeCl_3$, 0.25 for $CaCl_2$, and 0.11 for NaCl. This expulsion of acid occurs regardless of the fact whether or not the membrane is coated with gelatin, as a comparison of Experiment 2 with Experiment 5 shows. After 3 hours the difference in acid on the opposite sides of the membrane becomes less and finally disappears. In the case of Na_2SO_4 this effect is not noticeable.

When the writer first observed these phenomena he was under the impression that they were due to the action of the membrane, and he expressed this belief in a paper published a year ago.[1] Recent experiments to be mentioned presently have convinced him that the membrane has little if anything to do with this phenomenon and that it finds its explanation in the influence of salts on the diffusion constant of acids discovered by Arrhenius and described by him as follows:

"The theoretical study of the phenomenon of diffusion has led to a conclusion which seems very paradoxical. If hydrochloric acid diffuses in water, its diffusion constant is found to be 2.09 at 12°C., which also agrees very well with the theory of Nernst. If instead of using pure water for the diffusion, I take a solution of sodium chloride, I might expect that the molecules of HCl moved, *i.e.*, diffused more slowly in that medium than in water because of the increase of the viscosity on addition of NaCl. But instead of that an increase of the constant of diffusion is observed. For instance into a cylindrical vessel was poured a layer of 1 cm. height of 1.04 *n* HCl and over it was placed pure water to a height of 3 cm. The diffusion constant was found to be 2.09 at 12°C. In another experiment 0.1 *n* NaCl was used instead of water, so that at the bottom of the cylindrical vessel was a 1 cm. high layer 1.04 *n* in regard to HCl and 0.1 *n* in regard to NaCl and over it were placed 3 cm. of 0.1 *n* NaCl.

"The diffusion constant was now 2.50. According to Nernst's theory I calculated 2.43. In 0.67 *n* NaCl the constant was still higher 3.51, calculated 3.47. Many analogous experiments with results agreeing with the dissociation theory were performed with nitric and hydrochloric acid, caustic potash and soda.

"The explanation is that when the H-ions (*i.e.*, the acid) diffuse in pure water they must drag the (about 5 times) more immobile Cl-ions with them in equivalent number. If now Na-ions are distributed in the same fluid, these ions are carried back in the opposite direction of the diffusing H-ions because of the electric forces which hold back the H-ions and pull on the Cl-ions in the direction of diffusion (upwards in the experiments). The driving back of the Na-ions partly neutralizes these electric forces, so that therefore the H-ions are not so strongly held back nor the Cl-ions pushed up as in pure water. Therefore the H-ions diffuse more rapidly and that in a so much higher degree as the Na-ions are more numerous relatively to the H-ions. The maximal velocity which the H-ions may reach at 12° is that corresponding to no hindrance from electrical forces and gives a diffusion constant 6.17. With 0.52 *n* HCl and 3.43 *n* NH_4Cl I reached a value of 4.67 instead of theoretically calculated 5.72.—It must be borne in mind that at these high concentrations the degree of dissociation of the HCl is diminished, which is not taken into consideration, and therefore the observed dissociation constant is smaller than the calculated one determined with the supposition that the electrolytic dissociation is complete.

"This phenomenon is a so-called salt action, which can not be explained if we suppose the molecules of the diffusing acids or bases and the added salts not to be dissociated into electrically charged ions. It is a real proof of the electrolytic dissociation hypothesis."[2]

On the basis of Arrhenius' statement, the phenomenon observed by the writer might find its explanation in the following way. When a solution of, *e.g.*, M/1,000 $CeCl_3$ is made up in HCl so that the pH is 3.0, and if this solution is put into a collodion bag of 50 cc. volume, which is immersed in a beaker containing pure HCl (without salt) also of pH 3.0, the osmotic pressure of Cl ions is about four times greater in the salt solution than on the opposite side of the membrane. Hence four times as many Cl ions will diffuse through the membrane from the salt solution into the water as will diffuse simultaneously in the opposite direction. H and Ce ions will be dragged along electrostatically with the moving Cl ions, but on account of their greater mobility H ions will follow the Cl ions more readily than will the slow Ce ions. As a consequence the hydrogen ion concentration will rise on the water side of the membrane and diminish on the side of the salt solution; but on account of the fact that the volume of water was seven times as great as that of the salt solution inside the collodion bag, the change in pH was more marked in the salt solution inside the bag than outside. If this explanation is correct, the membrane itself has nothing to do with the phenomenon except to make it easier to demonstrate the salt effect on the rate of diffusion of the acid. If this conclusion is correct, it should be possible to prove that the rate of diffusion of acid into water through a membrane is accelerated through the addition of salt and the more the higher the concentration of the salt.

This proof was furnished in the following way. 350 cc. of different concentrations of NaCl, $CaCl_2$, $LaCl_3$, or Na_2SO_4 were made up in HCl so that each solution was N/2,000 in regard to the acid. Into these mixtures of N/2,000 HCl and salt were dipped collodion bags with a volume of about 50 cc. filled with water of pH 4.7. In this case the salt solution was outside the bag and the water free from salt was

[2] Arrhenius, S., Theories of solutions, New Haven, London, Oxford, 1912, 163–165.

inside the collodion bag. Both salt and acid diffused into the bag. After 20 minutes the collodion bags were taken out, the volume of liquid measured, and 10 cc. of the solution were titrated with N/400 NaOH to pH 5.4, using methyl red as an indicator.

In these experiments the concentration of the acid outside did not change materially since the volume of the outside solution was seven times as great as the inside solution, and the volume of the liquid inside the bag also remained practically the same except at the higher concentrations of the salt where the osmotic effect became noticeable. We may therefore use the titration value for 10 cc. of the content of the bag as a measure for the rate with which acid diffuses from the outside solution into the collodion bag. These titration values are plotted in Fig. 1 as ordinates over the concentrations of the salts as abscissæ. 0.25 cc. of N/400 NaOH were required to bring the acid contained in 10 cc. of the contents of the collodion bag to pH 5.4 when no salt was present. If we call this rate for the diffusion of acid through the membrane 1, then it is seen that the rate of diffusion of HCl is doubled when the concentration of NaCl is between M/64 and M/128, when the concentration of $CaCl_2$ is between M/128 and M/256, and when the concentration of $LaCl_3$ is about M/512 or perhaps a little below. It is obvious that the effect of these three salts on the rate of diffusion of HCl through the membrane increases with the concentration of the Cl ions and that all three salts accelerate the rate of diffusion of HCl through the membrane. H_2SO_4 diffuses more slowly through the membrane than HCl, since the titration value for acid without salt is 0.20. This rate of diffusion is doubled when the concentration of Na_2SO_4 is between M/256 and M/128. At higher concentrations both Na_2SO_4 and $CaCl_2$ show anomalies which need not be discussed in this connection.

These experiments were open to the objection that the pH of the N/2,000 HCl solution was different in $LaCl_3$, $CaCl_2$, or NaCl and that this caused the difference in the influence of the three salts on the rate of diffusion. Measurements of the pH showed that it was practically the same in all three solutions and had therefore nothing to do with the result. This was, however, not true in the case of mixtures of H_2SO_4 and Na_2SO_4 (as had already been found by Thomas and Baldwin[3]), and for high concentrations of $CaCl_2$. These interesting phenomena can, however, not be discussed in this connection.

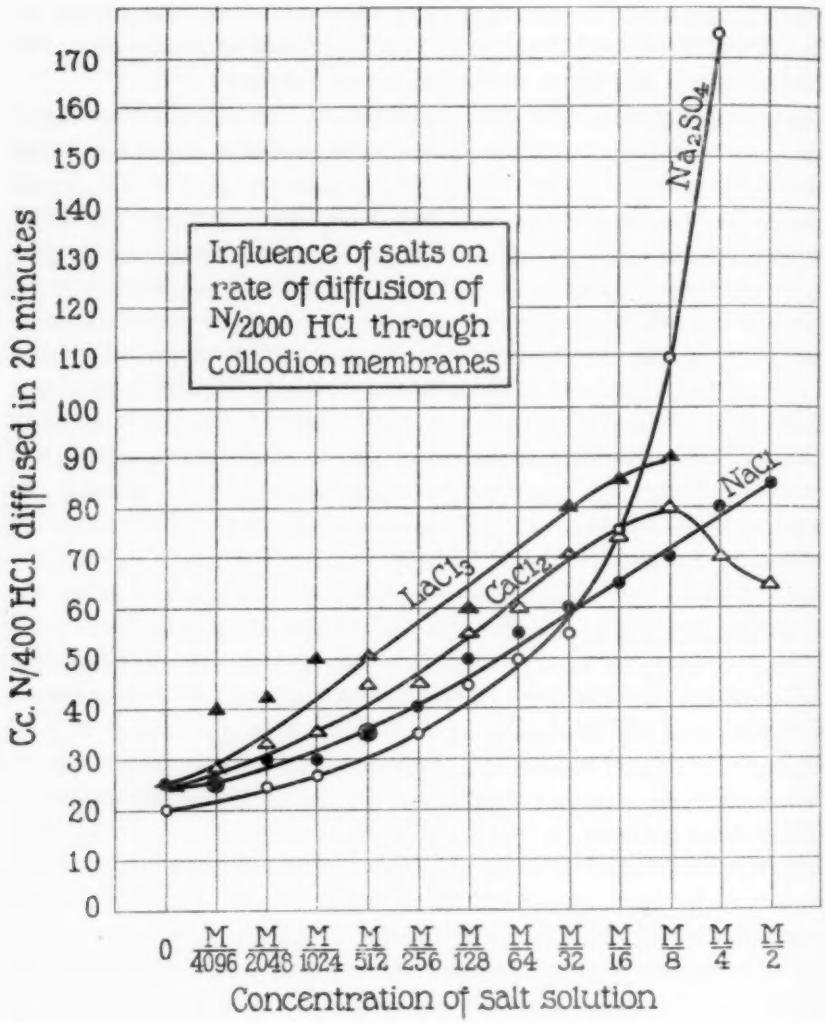

FIG. 1. Influence of salts on rate of diffusion of N/2,000 HCl through collodion membranes. Abscissæ are the concentrations of salts; ordinates, cubic centimeters of N/400 NaOH required to bring 10 cc. of water inside to pH 5.4 after 20 minutes of diffusion.

In a series of papers and a recent book the writer has tried to prove that the influence of electrolytes on the colloidal properties of protein solutions, such as osmotic pressure, viscosity, membrane potentials, is merely the consequence of the Donnan equilibrium. The equilibrium rests on the fact that many membranes which are freely permeable for small crystalloidal ions are impermeable for protein ions. The writer has shown that proteins behave in regard to chemical reactions like crystalloids and the same seems to be true as far as solubility is concerned. This then leads to the conclusion that the only reason for the colloidal behavior of protein solutions in regard to osmotic pressure, viscosity, etc., lies in the non-diffusibility of proteins through membranes and gels which are permeable to crystalloids. If we could produce membranes permeable to Na but not to SO_4 ions it would be found that a Na_2SO_4 solution would show colloidal behavior in regard to osmotic pressure and membrane potentials. This postulate of the theory of colloidal behavior is met in the facts published in this paper. It is not necessary that the membrane should be completely impermeable for one ion; it suffices that the rate of diffusion of one type of ions be considerably slower than that of another kind. The slow ions are the cations of salts, especially the polyvalent cations.

These experiments were undertaken to find out whether the influence of salts on the rate of diffusion of acid through the membranes of living cells was the same as through collodion membranes. This is not the case since salts retard the rate of diffusion of acid through membranes of living cells instead of accelerating it. This retarding effect of salt on the diffusion of acid—the so called antagonistic salt effect—was observed by Loeb and Wasteneys in the case of the marine fish *Fundulus*.[4] Thus the fish died in 3 M/10,000 HNO_3 in less than an hour while they lived indefinitely when the same HNO_3 solution was made up in M/10 NaCl. $CaCl_2$ was much more effective in the prevention of the toxic effects of acid. Later the same inhibiting effect of salt on the toxic effect of acid was observed in the eggs of *Fundulus*.[5]

[3] Thomas, A. W., and Baldwin, M. E., *J. Am. Chem. Soc.*, 1919, xli, 1981.
[4] Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxiii, 489; 1912, xxxix, 167.
[5] Loeb, J., *J. Biol. Chem.*, 1915, xxiii, 139; 1917, xxxii. 147.

Since the effect of salts on the diffusion constant of acids must be the same in the case of membranes of living cells as in the case of collodion membranes, the retarding effect of salts on the diffusion of acids in the egg of *Fundulus* can only be ascribed to a modification of the membranes of *Fundulus* by the salts whereby a block is created to the diffusion of acids.

SUMMARY AND CONCLUSION.

1. The writer had previously published the observation that if a salt solution made up in an acid solution (*e.g.* HCl) of a definite pH (*e.g.* 3.0) is separated by a collodion membrane from pure water containing the same acid of the same pH, acid is at first driven from the salt solution into the water, so that the pH of the latter becomes at first lower than that of the solution.

2. It is shown in this paper that this paradoxical phenomenon is not due to any peculiarity of the membrane but is a consequence of the well known fact that the diffusion constant of an acid is increased by a salt.

THE MECHANISM OF THE INFLUENCE OF ACIDS AND ALKALIES ON THE DIGESTION OF PROTEINS BY PEPSIN OR TRYPSIN.

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It has long been known that pepsin digestion occurs only in acid solution while trypsin digestion takes place in neutral or alkaline solutions. This marked influence of the reaction on the rate of hydrolysis is a characteristic property of enzymes in general. A great advance in the explanation of this phenomenon was made by Sörensen (1) who was able to show in the case of a number of enzymes that the controlling factor was the hydrogen ion concentration, and that the other ions present were of secondary importance. Michaelis (2) found that the curves expressing the rate of hydrolysis as a function of the pH could be accurately calculated on the assumption that the enzyme was a weak acid or base, and that the activity was due either to the ion or to the undissociated molecule, depending on the nature of the enzyme. Michaelis' experiments leave little doubt that the experimental curve is very similar to a dissociation curve. The weak point in the hypothesis is the lack of direct or independent evidence for the ionic character of the enzymes and the fact that migration experiments (3) on pure pepsin appear to contradict some of the assumptions used in the calculations. It had been suggested by Euler (4) and by Arrhenius (5) that the effect of the acid was upon the substrate rather than on the enzyme. The writer was able to show (6) that in the case of pepsin this mechanism served very well to explain the results, since it was found that the rate of hydrolysis could be quite accurately predicted from a measurement of the concentration of ionized protein and also that the pH for minimum digestion shifted with the isoelectric point of the protein. This indicates that the effect is primarily on the protein. The present paper contains

the results of experiments designed to test this assumption more fully and to extend the observations to trypsin. It has been found that the rate of digestion of gelatin, casein, and hemoglobin by pepsin or trypsin may be predicted from the amount of protein ion present. The acid salt of these proteins is rapidly attacked by pepsin, and the alkali salt by trypsin. The curve for the rate of digestion plotted as a function of the pH is therefore nearly identical with the titration (dissociation) curve of the proteins. The same result has been found with trypsin, edestin, and globin in that the alkaline titration curve of these proteins agrees with the curve for the rate of trypsin hydrolysis.

Experimental Procedure.

Regulation and Measurement of the pH.—The measurements were made with the gas chain at 33°, using 0.10 N HCl as the standard. The pH of this acid was taken as that given by the conductivity ratio: 1.036.

Buffers.—In the pepsin experiments no buffer was used; the protein solution was simply titrated to the desired pH with acid (HCl). There was no significant change in pH during the reaction. Trypsin hydrolysis causes a very large change in the pH and it is necessary to use a powerful buffer. It was found that a combination of phosphate, borate, and citrate would furnish a solution having a high buffer value over a wide range of pH. It has the further advantage that the nature of the ions does not vary. The titration curve of this buffer is given in Fig. 1. It is not intended to be used as a standard and for accurate values the solutions should be checked in some way. The final concentration of the buffer was usually one-quarter that of the original solution.

Pepsin.—The pepsin used was a sample of specially active Fairchild's pepsin (U.S.P. 1:20,000).

Trypsin.—Fairchild's trypsin was used. It was purified by dialysis, as described in a preceding paper (7).

Temperature.—All experiments were made at 33° ± 0.5°C.

Determination of the Rate of Digestion. Pepsin. Gelatin.—The rate of hydrolysis was determined by noting the time for a sample to reach a definite degree of liquefaction, as previously described (6).

Casein.—The casein (8) was dissolved in dilute NaOH and brought to the required pH by addition of the appropriate buffer. An aliquot portion of the solution was removed, brought to approximately pH 4.7 and added to 3 volumes of acetate buffer pH 4.7. The precipitated casein was then filtered, washed with water, dried at 100° and weighed. The curves obtained in this way were plotted and the time required to dissolve about 10 per cent of the total casein read off. This value has been taken as the reciprocal of the velocity of hydrolysis.

Hemoglobin.—The hydrolysis was followed by means of the increase in amino nitrogen, by the Van Slyke method (9).

Trypsin. Casein.—The same method was used as that described above. The hydrolysis was also followed by the Van Slyke method for amino nitrogen.

Gelatin.—The hydrolysis was followed by the increase in formol titration (10) and also by the Van Slyke method.

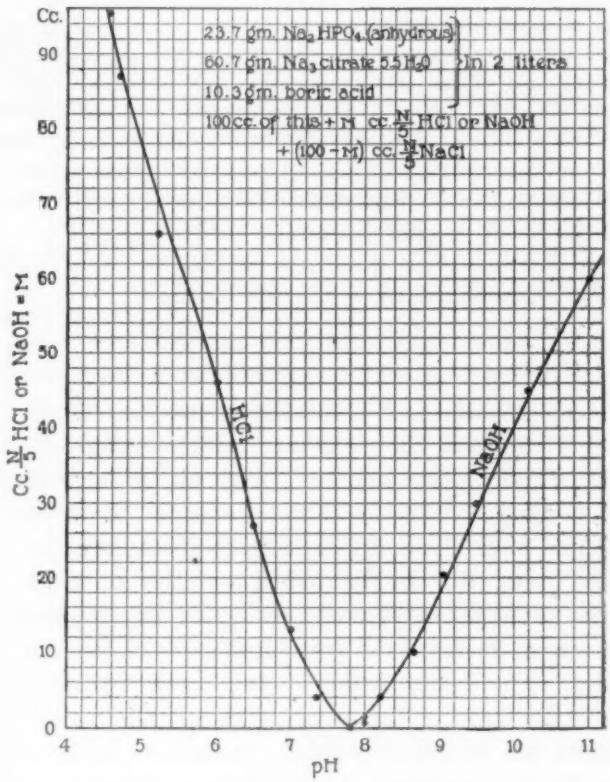

FIG. 1

Globin.—The globin was prepared as described by Robertson (11). The rate of hydrolysis was followed by bringing the solution to the isoelectric point (about pH 6) and filtering off and weighing the undissolved protein.

Edestin.—The writer is indebted to Dr. Hitchcock for the edestin used in this experiment. The rate of hydrolysis was determined by the Van Slyke method and also by precipitating and weighing the unattacked protein.

Titration Curves.—The titration curves were determined by adding a known amount of alkali or acid to the protein solution and determining the pH. A similar series was carried out in which the protein was omitted. The amount of protein combined with the acid (or alkali) was then determined by subtracting the cubic centimeters of acid required to bring water to any given pH from the cubic centimeters of acid required to bring an equal volume of the protein solution to this pH. This correction is negligible between pH 3 and 9. The curves obtained were identical with those given by Loeb.

In the case of casein solution it was found that two entirely different pH curves were obtained (in the region near the isoelectric point), depending on whether the supernatant fluid or the whole suspension was measured. This behavior had also been noted by Cohn. If the measurement was made on the whole suspension, the curve rises much more steeply near the isoelectric point and is similar to the curve given by Van Slyke and Baker (8). It also resembles closely the curve for gelatin and does not conform to the typical titration curve. Loeb (12) has recently shown that this effect is due to the fact that the pH of a suspension is intermediate between that of the supernatant liquid and that of the particle. The correct curve is that of the supernatant fluid.

Conductivity measurements (6) show that the protein salt is completely dissociated at least until a very large excess of the common ion is added. The titration curve, therefore, gives directly the amount of ionized protein up to the maximum value; beyond this point the amount of ionized protein decreases whereas the amount of combined acid remains constant. This decrease in the ionized protein can be followed in the case of gelatin (on the acid side) as described in a previous paper (6). In the case of the other proteins, however, the point lies in the region of such strong acid that the measurement is very uncertain, owing to the very large value of the correction for the free acid. The determination of this value was, therefore, not attempted in the case of the other proteins. On the alkaline side the conditions are more complicated, owing to the fact that there are apparently two alkaline salts formed, *i.e.*, there is a group of acid radicals that is completely neutralized at pH 9 and another group that is not neutralized until a pH of 11 or 12 is reached. This is very marked in the case of gelatin and casein. Hemoglobin, however, shows only a very slight flattening of the curve at pH 7.5; the second group of acid radicals evidently begins to combine with the alkali before the first group is completely neutralized.

Effect of the Amount of Enzyme.—It has been found by the writer (13) and also by Ringer (14) that trypsin is very rapidly destroyed in solutions of pH greater than 10. In order to reduce this effect, it is necessary to use relatively large amounts of trypsin so that the experiment is completed in as short a time as is permitted by the experimental limitations. If this is not done the results are complicated by the influence of the pH on the trypsin itself. This effect would evidently cause the optimum to shift to the acid side and would be more marked the more dilute the trypsin; *i.e.*, the longer the experiment is allowed to run. Several experiments were made to test this point and it was found that, with the amounts of trypsin used in these experiments, the position of the optimum was independent of the amount of trypsin used. The drop in the rate of digestion in strong alkali, therefore, cannot be attributed to the destruction of the trypsin by the alkali. This complication does not enter in the case of pepsin, since this enzyme is stable, even in strong acid.

Presentation of Results.—For the sake of brevity the original curves obtained for the increase in the digestion with time are not given, but only the rates interpolated from these curves. The results were plotted on a large scale and the time (in hours) required to hydrolyze 10, 20, and 30 per cent of the protein read off from the curves. The reciprocal of these times is taken as a measure of the rate of digestion. It was found that the values obtained in this way were the same, within the rather large experimental error, independent of the percentage hydrolysis which was used as the end-point. In other words, the rate of hydrolysis-pH curve is independent of the stage of hydrolysis, in the beginning. If the results are taken from a much later stage of hydrolysis, the curve is changed slightly, as would be expected, since the secondary reactions become more predominant and the effect of the pH on the destruction of the enzyme more pronounced. No significant effect of different enzyme concentration could be noted, nor did the different methods of following the hydrolysis give results which differed more than the experimental error. It is unnecessary, therefore, to distinguish between the different experiments.

In order to reduce all the experiments to a comparative basis, the results have been calculated to a percentage scale; *i.e.*, the maximum rate of hydrolysis has been taken as 100 and the rates at other pH expressed as percentages of this maximum.

Comparison of the Titration Curve and the Rate of Hydrolysis.—In order to compare the rate of hydrolysis with the amount of protein salt found, it is necessary to express both quantities in the same units. This has been done by calculating the titration curve also to a percentage basis. The largest amount of alkali or acid which is found to combine with the protein has been taken as 100 and the amounts at other pH expressed as per cent of this figure. The maximum on the acid side is well defined and there is no difficulty in deciding at which pH the protein is all present as the acid salt. On the alkaline side, as mentioned above, the titration curve shows that there are two alkaline salts formed. It could not be told *a priori* from the titration curve alone which of the salts was attacked by the trypsin. Comparison with the hydrolysis pH curve, however, shows that, except in the case of globin, the maximum rate of hydrolysis occurs at the same pH as does the maximum formation of the *first* alkali salt. The point at which the greatest amount of this salt is formed has therefore been taken as 100. In the case of globin the hydrolysis increases with the second rise in the titration curve, indicating that in this case the second salt formed is still more easily attacked. The second maximum in the titration curve has therefore been taken as 100 in the case of this protein. The position of this maximum is much more difficult to determine and it is possible that the quantitative agreement of the titration and rate of hydrolysis curves of globin is partly due to an incorrect value for the maximum point of the titration curve. There is no doubt, however, that the curves agree qualitatively in that they both have a flat portion between pH 8 to 9, followed by a second rise; the only uncertainty is as to the exact coincidence of this second maximum. The hydrolysis curve cannot be carried further, owing to the destruction of the trypsin.

Results of the Experiments. Influence of the Ions Other Than H and OH and the Influence of the Physical Properties of the Substrate.—The work of Sörensen (1), Michaelis and his coworkers (2), and of the writer (6, 13, 15) has shown conclusively that the hydrogen or

hydroxyl ion is the determining factor and that the nature and valence of the other ion is of very minor importance as is the physical condition of the substrate. Nevertheless the question of the influence of the "degree of hydration" or swelling of the protein has again been raised by Ostwald (16). This idea may be traced back to Brücke (1859). Brücke (17) was a pioneer worker and it is easy to understand his suggestion that swelling and digestion are connected since both increase with increasing amounts of acid. At present, however, it would seem to be difficult to add anything to the proof already existent that the swelling, osmotic pressure, "degree of hydration," etc., have no direct connection with the hydrolysis of proteins by either pepsin or trypsin. Since, however, this is a fundamental point in the study of these enzymes, some of the experiments referred to were repeated. The critical experiment is to compare the rate of hydrolysis of a protein in the presence of sulfate and chloride ion and in the presence of Na and Ba. It has been shown by Loeb (18) that the osmotic pressure, viscosity, and swelling of a protein are very much less in the presence of bivalent ions than in the presence of monovalent ions at the same pH. It has also been shown that the amount of acid required to bring the protein solution to a given pH is independent of the nature and valence of the anion. In other words the chemical effect of all acids is the same, but the effect on the physical properties is different for various acids. It has also been shown by Loeb that this effect on the physical properties has nothing to do with the degree of hydration, but is the result of an equilibrium governed by the law of mass action, the theory of which was stated by Donnan (12). If the rate of hydrolysis, then, depends on any physical property of the protein, there should be a marked difference in the rate of hydrolysis of gelatin in the presence of sulfuric and of hydrochloric acid. The results of an experiment made to test this point are shown in Fig. 2. The figure shows the rate of hydrolysis of gelatin by pepsin in the presence of sulfuric acid and of HCl, and of gelatin and casein by trypsin in the presence of Ba and Na. As the figure shows, there is no difference whatever in the rate of hydrolysis in sulfuric acid as compared to HCl solution nor in $Ba(OH)_2$ as compared with NaOH (of the same pH), although the swelling, viscosity, and osmotic pressure are very much less in the presence of the divalent ions.

In the case of two solutions in one of which the protein is soluble and in the other insoluble, it would be expected that there would be a difference in the rate of digestion, no matter what mechanism is assumed to govern the reaction, since the concentration of the protein in the solution is entirely different in the two cases. In the insoluble case the protein salt is concentrated at the bottom of the vessel while in the soluble case it is distributed throughout the solution.

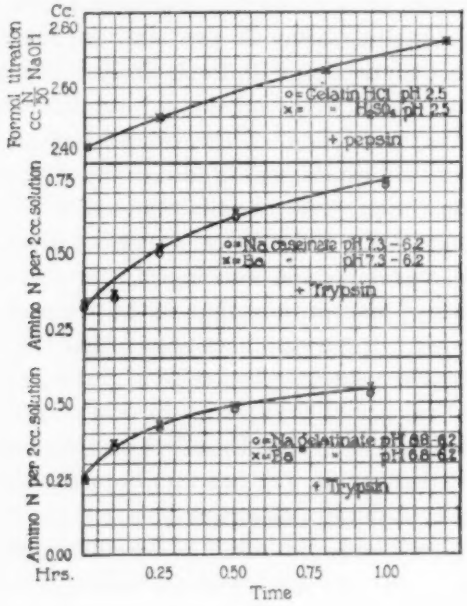

FIG. 2

Even here, however, there is no striking difference in the rate of digestion, as shown by the writer's experiments (19) with edestin and casein in hydrochloric and sulfuric acids and Gyemant's (20) experiments with sulfosalicylic acid.

Relation between the Titration Curve and the Rate of Hydrolysis.—The experiments described above show that the effect of the acid and alkali is primarily on the chemical condition of the protein. Increasing addition of acid or alkali causes the formation of increasing amounts of protein salt. The increase in this amount of salt is shown

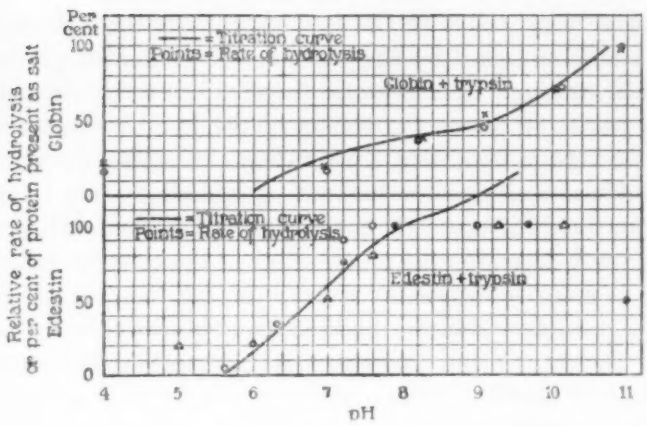

FIG. 3

by the titration curve. If the increase in the rate of hydrolysis is due to the fact that the enzyme reacts with the ionized protein, the increase in the rate of hydrolysis with increasing acid or alkalies should be identical with the titration curve. The results of the experiments are shown in Figs. 3 and 4. The figures show that in general the

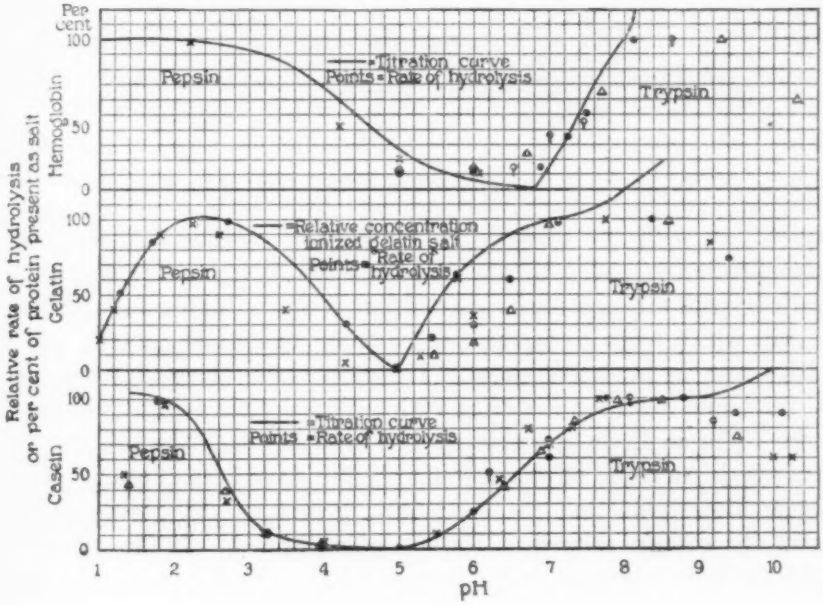

FIG. 4

results agree very well with the assumption that the ionized protein is more readily attacked by the enzyme. The agreement in the case of casein is striking. In every case the solid line is the titration curve and the points represent the results of the hydrolysis experiments. It must be kept in mind that the titration curves give the amount of acid combined and not the amount of ionized protein. The two figures are the same up to the maximum, but beyond that point the ionization will decrease, due to the effect of the common ion. This, decrease is actually shown in the case of gelatin in acid solution where it is possible to measure the ionization by conductivity measurements. The experiment shows that this decrease is in quantitative

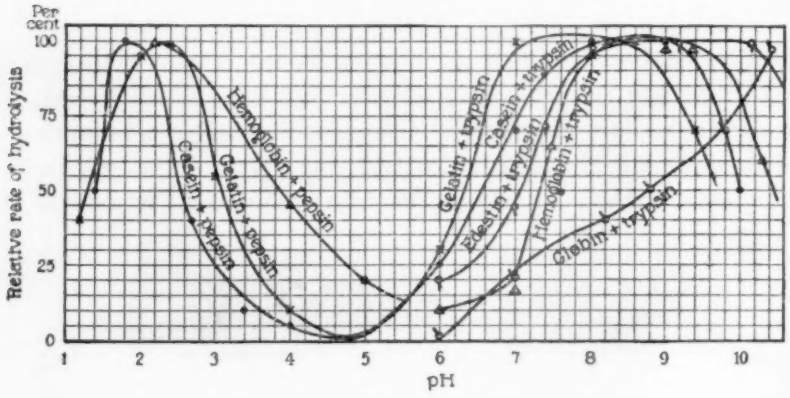

FIG. 5

agreement with the decrease in the rate of hydrolysis. Another complication is added by the fact that the hydrolysis experiments with trypsin are necessarily carried out in fairly high concentration of salt which tends to decrease the amount of ionized protein, especially near the isoelectric point. The curves also show that the pH of minimum digestion is shifted with the isoelectric point of the protein. This is shown more clearly in Fig. 5 in which the pH rate of hydrolysis curves of all the proteins studied have been plotted. The figure also shows that the descending branch of the curves varies with the protein as well as the ascending branch. If the decrease in hydrolysis found on adding excess alkali or acid were due to the

effect on the enzyme, as has been proposed by Gyemant (20) it would be expected that the descending curves would all be the same.

The experiments as a whole substantiate the idea that the reaction of the protein and enzyme depends on the concentration of ionized protein, although the experiments do not all show quantitative agreement with this mechanism. Pepsin reacts with positively charged protein ions and trypsin with negatively charged protein ions. This view is also supported by the fact that when the protein is insoluble, it combines with pepsin only when the protein is positive (21). It is also in agreement with the result of Pekelharing and Ringer (3) who found that pure pepsin is negatively charged.

A marked difference between the rate of reaction of ionized and unionized substances is the general rule in all chemical reactions and has been found to be at the basis of many reactions; it is not surprising, therefore, that it should be found to hold also in the case of protein hydrolysis. It may be remarked that the writer (10) has found that in the case of gelatin hydrolyzed by acid or alkali without any enzyme, the relative ease of hydrolysis is reversed and the results can be accounted for on the assumption that the unionized gelatin is more rapidly attacked.

An objection to this point of view which presents itself is the fact that in the case of sugar and other substances, it can hardly be supposed that the acid or alkali could affect the substrate in such a way as to give a titration curve. Michaelis and Rothstein (22) have recently suggested that in the case of sugar, a compound is formed between the invertase and the sugar, and that it is the dissociation of this compound that regulates the hydrolysis.

SUMMARY.

1. The effect of the addition of acid on the amount of ionized protein has been compared with the effect on the rate of digestion of gelatin, casein, and hemoglobin by pepsin.

2. A similar comparison has been made of the addition of alkali in the case of trypsin with gelatin, casein, hemoglobin, globin, and edestin.

3. In general, the rate of digestion may be predicted from the amount of ionized protein as determined by the titration curve or

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conductivity. The rate of digestion is a minimum at the isoelectric point of the protein and a maximum at that pH at which the protein is completely combined with acid or alkali to form a salt.

4. The physical properties of the protein solution have little or no effect on the rate of digestion.

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